# Epigenetic Response and Adaptation to Salt Stress in *Arabidopsis thaliana*

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A thesis submitted in partial fulfilment of the

requirements for the degree of

**Doctor of Philosophy in Life Sciences**

University of Warwick, Department of Life Sciences

January 2016

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## ABSTRACT

High soil salinity is a major environmental stress that adversely affects crop production throughout the world. It is now estimated that half of the world's cropland is affected by salt stress. To cope with various environmental stresses, plants are able to spatially and temporally regulate gene expression through changes in DNA methylation and chromatin conformation, known as epigenetic modifications. Recent studies indicated that epigenetic modifications induced by environmental stress can be inherited over several generations, despite a genome-wide epigenetic resetting of epigenetic imprints that takes place during plants reproduction. In this thesis, I evaluated in *Arabidopsis thaliana* the effect of multi-generation salt stress treatments on the genome-wide dynamics of DNA methylation and tolerance to high salinity. My results show that the immediate progenies of stressed plants displayed better germination and survival rate under high salinity, but contrary to current theories this effect is lost in the following non-stressed generation. Genome-wide DNA methylation analysis revealed that stress induced discrete *de novo* methylation and demethylation changes on epigenetically labile regions of the plant genome. These acquired tolerance and methylation marks are likely under parent-of-origin control as a result of a robust epigenetic reprogramming that takes place in the male germline. Stress-induced methylation marks identified are associated with transcriptional changes of stress responsive genes and correlated with antisense long-non coding RNA expression. Overall this work establish for the first time a link between differential DNA methylation, gene expression and short-term adaptation to stress in plants.

# Declarations

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. The work presented in this thesis is original, and has not been published or presented for any other degree. The work described in this thesis has been carried out by myself, with the exceptions described below:

**Dr. Jörg Becker (The Instituto Gulbenkian de Ciência, Portugal)**

Assisting purification and isolation of Vegetative Nuclei or Sperm Nuclei by Fluorescense Activated Cell Sorting (FACS) (Chapter 4).

**Dr. Gianpiero Marconi (University of Perugia, Italy)**

Assisting qRT-PCR gene expression analysis (Chapter 5).

**Dr Claude Becker (Max Planck Institute for Developmental Biology, Germany)**

Produced bisulphite converted Illumina libraries and assisting the bioinformatics analysis of the datasets (Chapter 4).

## Acknowledgements

First, I would like to thank Professor my supervisor, Dr. Jose Gutierrez-Marcos for his constant guidance and support throughout this research, I also thank him for the opportunity to develop research ideas into project and for encouraging critical thinking. I would like to express my gratitude to our collaborator, Dr. Claude Becker and Professor Detlef Weigel for their assistance, support and valuable suggestions. I would also like to thank my PhD advisory panel Professor Jim Beynon and Dr. Miriam Gifford, and my PhD Thesis examiners Dr. Vardis Ntoukakis and Professor Anna Amtmann for their time and effort in reviewing my PhD works and for their valuable feedback. My gratitude is extended to Mr. Gary Grant for his laboratory support and for colleagues who has provided me with research materials and technical advices.

Many thanks to everyone at Marcos group, past and present, especially for Mr. Ranjith Paparedy for years of helps and sincere company, also to Dr. Gianpiero Marconi, Dr. Julius Durr, Mr. Quentin Saintain, Mr. Jonathan Price, Mr. Hadi Lanang Putra, Ms. Shannon Easterlow for their helps and support throughout the research. Finally, my heartfelt thanks to my family, my parents Mr. Djumpari and Mrs. Roch Supadmi for their love and encouragement, my wife Fitria Nungky Harjanti for her assistance in the lab and for her constant love and patience, and for my daughter Hanum Kinanthi Wibowo, my source of happiness and motivation.

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## List of Abbreviations

| | |
|---|---|
| 5mc | 5-methylcytosine |
| ABA | Abscisic Acid |
| ABRE | ABA-Responsive Element |
| amiRNA | artificial microRNAs |
| BABA | β-amino-butyric acid |
| bHLH | basic Helix–Loop–Helix |
| Bp | base pair |
| CDS | Coding DNA Sequence |
| CIPK | CBL-Interacting Protein Kinase |
| DMP | Differentially Methylated Position |
| DMR | Differentially Methylated Region |
| DNA | Deoxyribonucleic Acid |
| dsRNA | double-stranded RNA |
| FACS | Fluorescence-Activated-Cell-Sorting |
| FSC | Forward Scatter |
| GFP | Green Fluorescence Protein |
| GO | Gene Ontology |
| GTL | GT element-binding-Like proteins |
| HAT | Histone Acetyltransferases |
| HDAC | Histone De-Acetylases |
| HMM | Hidden Markov Model |
| HS-DMRs | High Salinity-induced Differentially Methylated Regions |
| HSP | Heat-Shock Protein |
| ICP-MS | Inductively Coupled Plasma Mass Spectrometry |
| ISR | Induced Sytemic Resistance |

| | |
|---|---|
| JA | Jasmonic Acid |
| lncRNA | long non-coding RNA |
| LRR | Leucine-Rich Repeat |
| MA | Mutation Accumulation |
| MAPK | Mitogen-Activated Protein Kinases |
| MMC | Megaspore Mother Cell |
| MR | Methylated Region |
| MS | Murashige and Skoog |
| ORMV | Oilseed Rape Mosaic Virus |
| PCA | Principal Component Analysis |
| PMC | Pollen Mother Cell |
| PstDC3000 | *Pseudomonas syringae* pv *tomato* DC3000 |
| PTGS | Post Transcriptional Gene Silencing |
| qRT-PCR | quantitative Real-Time Polymerase Chain Reaction |
| RdDM | RNA-directed DNA Methylation |
| RFP | Red Fluorescence Protein |
| RNA | Ribonucleic Acid |
| ROS | Reactive Oxygen Species |
| SA | Salicylic Acid |
| SAR | Sytemic Acquired Resistance |
| SC | Sperm Cell |
| siRNA | small–interference RNA |
| smRNA | small RNA |
| SOS | Salt Overly Sensitive |
| SSC | Side Scatter |
| TE | Transposable Element |

| | |
|---|---|
| TGS | Transcriptional Gene Silencing |
| TMV | Tobacco Mosaic Virus |
| UTR | Untranslated Region |
| v/v | volume for volume |
| VN | Vegetative Nuclei |
| µg | Mikro-gram |
| µL | Mikro-liter |

# 1. General introduction

## 1.1 General Overview

Plants are sessile organisms that are constantly exposed to various environmental pressures. Because of this sessile nature, plants have to continually adjust to their environment, as not only are they exposed to one environmental stress at one single time, but they could be exposed to multiple stresses that occur at different intensities and durations. To cope with these variety of environmental stimuli and stresses, plants regulate their cellular and developmental processes through a network of complex responses (Atkinson and Urwin, 2012). When plants are exposed to stress, cells may perceive and memorize these stresses. This "stress memory" could modify their response to subsequent stresses within the same generation, which could make them better adapted to stress a process known as "priming" or "acclimatization" (Boyko et al., 2010; Hauser et al., 2011; Prime et al., 2006; Sani et al., 2013; Slaughter et al., 2012). In some cases, depending on the type, duration and intensity of the stresses, this stress memory may be passed down to the next immediate generation after stress or even over several generations after the initial stress treatment, a phenomenon called "transgenerational stress memory" (Hauser et al., 2011; Paszkowski and Grossniklaus, 2011). In the past, the study of "transgenerational stress memory" was often associated with Lamarck theory of evolution, where organisms can pass down specific characteristics or information that they acquired during their lifetime to the next generation (Pecinka and Mittelsten Scheid, 2012). This idea if often rejected because the lack of evidence and lack of mechanisms that could facilitate the encoding and inheritance of such information (Richards, 2006). However, a number of studies have clearly shown that DNA sequence is not the only carrier of information that determines the phenotype of an organism (Skinner, 2011). Novel regulation of gene expression, both spatial or

temporal, that leads to novel phenotype variations, can occur through dynamic modifications of DNA methylation, even in the absence of genetic variation. When organisms are exposed to environmental stress somatic cells may perceive and "memorize" the stresses in the form of epigenetic modifications. The majority of these stress-induced changes are reset to basal levels once the stress is alleviated, while some could be stably transmitted through the many rounds of mitotic and meiosis division of the germ cells and inherited to progenies (Chinnusamy and Zhu, 2009; Migicovsky and Kovalchuk, 2011).

The stability of epigenetic marks through mitotic cell divisions is well known in plants. However, during gametogenesis and embryogenesis, gametes and embryos undergo genome-wide epigenetic reprogramming where DNA methylation patterns are re-established and histone properties are extensively remodelled. This reprogramming is important in imprinting and required to ensure the totipotency and pluripotency of early embryonic cells (Grossniklaus et al., 2013; Gutierrez-Marcos and Dickinson, 2012). To be passed down to the next generation, stress-induced epigenetic modifications that are encoded on the parental somatic cells must be able to bypass this global epigenome reprogramming (Kawashima and Berger, 2014). Therefore, the epigenetic reprogramming and molecular mechanisms underlying this process have a key role in determining the sustainability of epigenetic inheritance across generations. Nevertheless, it remains unclear to what extent the epigenome reprogramming may limit the occurrence of transgenerational epigenetic inheritance and adaptation to environmental stresses. There are considerable number of studies demonstrating that epigenetic modifications induced by stresses can be passed down to the non-stressed generation. However, to date, there are no examples of transgenerationally inherited acquired trait, that are exclusively dependent upon

changes of the epigenetic state (Pecinka and Mittelsten Scheid, 2012).

In this chapter I will discuss the importance in plants of the epigenetic regulation mediated by DNA methylation and histone modifications, the epigenetic reprogramming taking place during gametogenesis and embryogenesis, and the relation between environmental stresses, epigenome reprogramming and transgenerational epigenetic inheritance.

### 1.2 DNA Methylation in Plants.

DNA methylation is a process conserved across various kingdoms of life. Although all DNA nucleotides can be methylated, the most common form of DNA methylation in higher organisms is cytosine methylation. It occurs through the covalent modification of cytosine with a methyl group at the 5' position, forming 5-methylcytosine (5mc) (Kalisz and Purugganan, 2004). In plants cytosine methylation could happen in different sequence contexts: symmetrical CG and CHG and asymmetrical CHH (where H is A, C or T). The *Arabidopsis thaliana* genome possessed methylation at 24% of CG, 6.7% of CHG and 1.7% of CHH sites (Cokus et al., 2008). DNA Methylation is widespread in plant's genome and can be found in all sequence motifs, however the preferred location for DNA methylation is at repetitive DNA sequences, which are commonly found at centromeres and Transposable Elements (TEs) (Gehring and Henikoff, 2008). Highly concentrated methylation on the repetitive DNA sequences suggests that one of the primary functions of DNA methylation is the suppression of transposon activity. TEs make up a substantial proportion of plant genomes, therefore the control of TE proliferation is necessary because they are potentially highly mutagenic and their

accumulation limits survival potential (Saze et al., 2012). Small amount of DNA methylation is also observed in gene coding regions in plants and often assembled in regulatory regions of genes such as promoter regions. Several studies have reported that methylation in the gene promoter causes reduced activity or even transcriptional silencing, suggesting that changes in methylation could lead to novel transcriptional regulation of the associated genes (Dowen et al., 2012; Du et al., 2015; Mette et al., 2000). In plants, cytosine methylation is established by a group of enzymes called DNA methyltransferases that transfer and attach methyl group into DNA. Symmetrical CG methylation is maintained through nuclear division by recognition of hemi-methylated daughter strands at the replication fork by VARIANT IN METHYLATION (VIM) family proteins. VIMs then recruit DNA METHYLTRANSFERASE 1 (MET1) and after DNA replication MET1 will transfer new methylation to the non-methylated daughter strands using hemi-methylated daughter strands as a template (Figure 1A) (Kawashima and Berger, 2014). Maintenance of CHG methylation also occurs during nuclear division and regulated by a feedback loop mechanism that involves CHROMOMETHYLASE 3 (CMT3), histone H3 lysine 9 di-methylation (H3K9me2), and SU(VAR) HOMOLOGUE 4 (SUVH4). This mechanism preferentially recognizes TE related sequences. CMT3 binds to H3K9me2, establishing methylation at CHG sites adjacent to it. The methylated DNA attracts SUVH4, a histone methyltransferase involved in H3K9me2 di-methylation. SUVH4 regulates H3K9me2 methylation and deposition around the CHG site, establishing CHG-H3K9me2 reinforcing feedback loop (Figure 1B) (Kawashima and Berger, 2014). On the other hand regulation of CHH asymmetric methylation requires a *de novo* process, as methylation is only found on a single strand before nuclear division. This process occurs via a RNA-directed DNA

Methylation (RdDM) pathway, which is unique to plants. This pathway involves the methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE 1 (DRM1) and DRM2 that are guided by small RNAs (smRNAs). The process is initiated by the binding of SAWADEE HOMEODOMAIN HOMOLOGUE 1 (SHH1) to H3K9me2, which then recruits Pol-IV and RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) to generate 24-nucleotide small RNAs. A complex made by ARGONAUTE proteins, 24-nt smRNAs, DRM1 and DRM2 then established *de novo* methylation at CG, CHG or CHH sites (Kawashima and Berger, 2014). An alternative pathway, which involves Pol II and RDR6 also exist in plants, generating 22-nt instead of 24-nt smRNAs. Recently, it has been shown that beside DRM1 and DRM2, another methyltransferase called CHROMOMETHYLASE 2 (CMT2) could also create *de novo* methylation at non-CG sites by directly binding to H3K9me2 (Figure 1C) (Kawashima and Berger, 2014). Unlike the maintenance of symmetrical methylation, *de novo* methylation via RdDM pathyway could facilitate formation of new methylation marks. In addition, secondary smRNAs generated during *de novo* methylation can also cause the further spreading of DNA methylation (Ahmed et al., 2011). Although DNA methylation is a stable epigenetic mark, it is also a reversible mark. DNA methylation can be actively removed by a process called DNA demethylation. Enzymes called DNA glycosylases facilitates the active removal of cytosine methylation through a base excision repair pathway. An example of DNA glycosylases in plants is DNA glycosylase DEMETER (DME) that is expressed at high-level in the companion cells of plant gametes, causing genome-wide demethylation and reactivation of some TEs. Other DNA glycosylases called REPRESSOR OF SILENCING1 (ROS1), DEMETER-LIKE 2 (DML2) and DML3 are expressed in various plants organs, and could facilitate small-targeted DNA

demethylation (Zhu, 2009). DNA demethylation could also occur passively. The passive DNA demethylations could take place when maintenance methyltransferase are inactive during several round of DNA replication, which result in loss of methylation following cell division (Zhu, 2009)

Disruption of DNA methylation has various effects on plants, including death. In *Arabidopsis thaliana,* homozygous *met1* mutant embryos had abnormality in cell division, both in embryo and suspensor cell. Genes that are normally expressed in embryo for regulating embryogenesis are misexpressed, and auxin gradient is not properly established in the *met1* embryo. The experiment using *met1cmt3* double mutant showed that double mutant plants have reduced seed size and viability compared to single mutant and wild-type plants (Xiao et al., 2006).

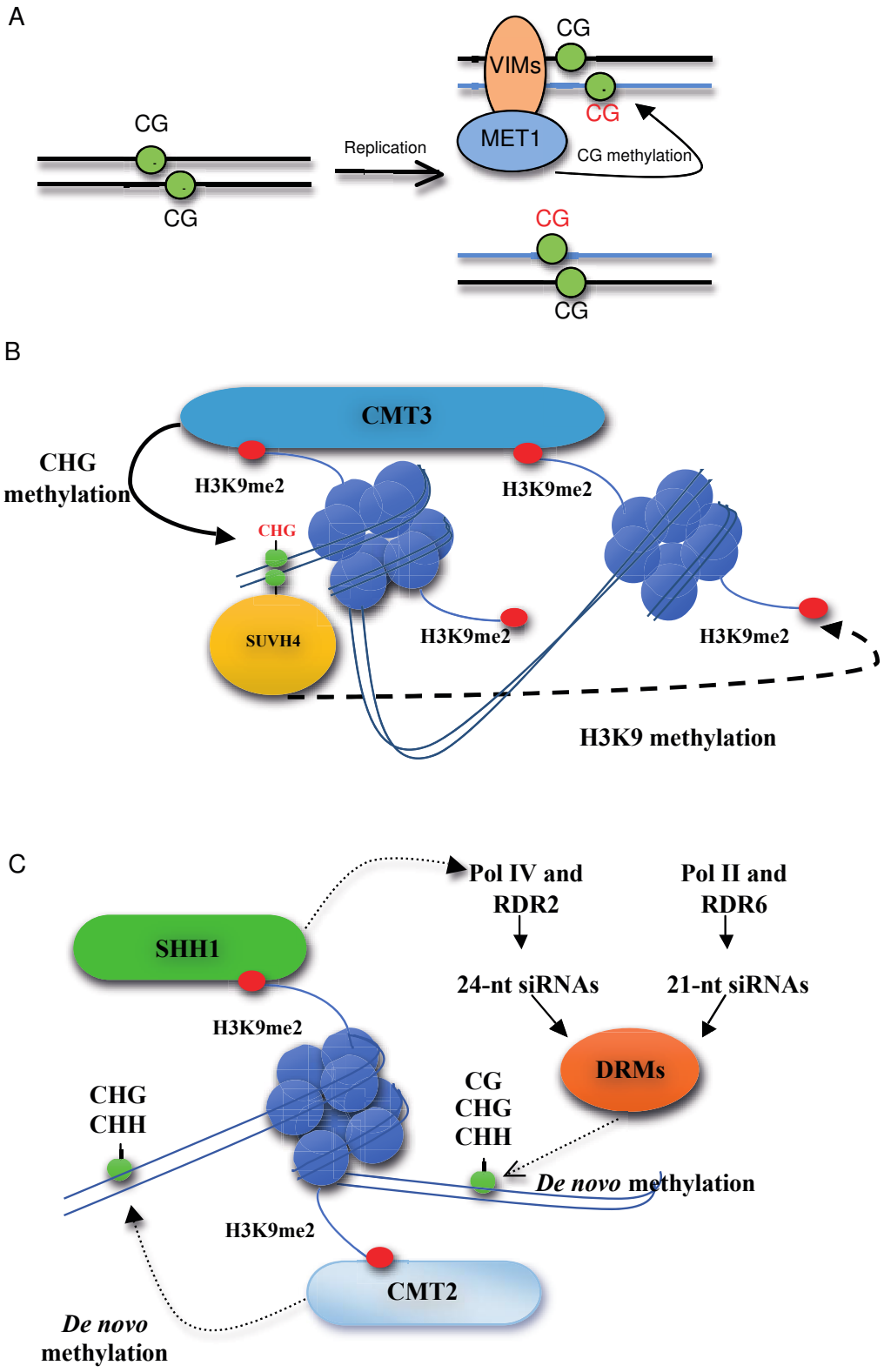


**Figure 1. Schematic diagram illustrating the maintenance of symmetric and asymmetric methylation**. (A and B) CG and CHG (symmetric) methylation is maintained through nuclear division by methyltransferases MET1 and CMT3, respectively (C) de novo (asymmetric) methylation is maintained by action of methyltransferases smRNAs guided DRM1 and/or by the action of CMT2 that directly bind to H3K9me2.

### 1.3 Histone Modifications in Plant

The remodelling of chromatin structure through the biochemical modification of histones is a main mechanism for epigenetic regulation. In eukaryotes, a complex of histone proteins called nucleoseome provide the core structures for chromatin packaging. The nucleosome is comprised of a histone protein octamer, consisting of two copies each of histone H2A, H2B, H3 and H4. This histone core is wrapped by approximately 147 bp of DNA to form nucleosome core particle. Beside the core histone, there is also the linker histone H1 and its isoforms that sits on the top of the nucleosome to keep the wrapped DNA strand around the nucleosome (Marks et al., 2001). Each histone subunit posseses a N-terminal tail which contains high number of basic amino acids such as lysine and arginine. This N-terminal tail extends away from the core particle and may be subjected to various post-translational modifications such as methylation, acetylation, phosphorylation, propinylation, formylation, citrullation, ubiquitylation, crotonylation, sumoylation, proline isomerisation and ADP ribosylation targeting lysine and arginine residues (Marks et al., 2001). Adding to the complexity, each of the amino acid residues at specific position in the histone-tail could be mono-, di- or tri-methylated or acetylated. Several studies had established that histone modification could specifically control the condensation level of chromatin and altering protein-DNA interaction (Bannister and Kouzarides, 2011).

The best-described histone modification is acetylation and methylation. Histones can be acetylated through the action of histone acetyltransferases (HATs) at specific lysine residues of histone H3, H4, H2A and H2B. Acetylation of histone is generally correlated with euchromatin (a more relaxed chromatin structure) and higher levels of gene transcription (Chen and Tian, 2007). Acetylation of histone N-tail could

alter the basic charge of the tail resulting in less condensed and transcriptionally active chromatin (Roth et al., 2001). Histone acetylation is a reversible modification and de-acetylation can occur via histone de-acetylases (HDACs). This can lead to suppression of expression as the chromatin is condensed from euchromatin to heterochromatin (a tightly packed form of DNA with limited transcriptional activity) (Roth et al., 2001).

Histone methylation usually occurrs at arginine and lysine residues. Arginine methylation is commonly associated with transcriptional activation, while lysine methylation has a more complex effect on transcriptional regulation (Liu et al., 2010). H3K4 and H3K36 methylation is often associated with transcriptionally active chromatin, for example a tri-methylated variant of H3K4 (H3K4me3) is often found at the promoter region of genes that are actively expressed (Cazzonelli et al., 2009). In the other hand, H3K9 and H3K27 methylation is often detected at transcriptionally silenced chromatin, for example H3K9me1 and H3K9me2 are often found at heterochromatic region, such as TEs and repetitive elements (Liu et al., 2010; Zhou et al., 2010).

Beside the histone code, the dynamics of histone modification is also important for fine-tuning of gene expression. For example mono- and di- methylation of H3K27 is associated with chromatin silencing, however tri-methylation of H3K27 can be found in both transcriptionally active and silenced chromatin (Lafos et al., 2011). In other cases, mono- and di-methylation of H3K9 are often associated with chromatin silencing (Xu et al., 2013), whereas tri-methylation of H3K9 is often found at early stage of transcription suggesting its role in genes activation. Interestingly, tri-methylation of H3K9 is quickly removed when transcription ends and the chromatin

is reverted back to a silenced state (Liu et al., 2010; Veiseth et al., 2011). The complexity of histone modification makes it necessary to differentiate the short and long term effects of histone modifications on gene transcription. It is also necessary to consider that each of modification at specific amino acid at specific histone type might interact and have combinative effect on gene expression.

### 1.4 Interaction between DNA Methylation and Histone Modifications in Plants.

Several studies have reported that DNA methylation could interact with histone modification to mediate transcriptional regulation in plants. A direct relation between DNA methylation and histone modification was showed in the regulation of CHG and CHH methylation, through interaction between CMT3 and RdDM pathyway with H3K9me2 (Kawashima and Berger, 2014). Interaction between DNA methylation and histone modification was also shown in the case of *ddm1* (Zemach *et* al., 2013), *kryptonite* (Habu et al., 2006), and *met1* (Soppe et al., 2002) *Arabidopsis* mutants. The DECREASE IN DNA METHYLATION (DDM1) chromatin remodeling factor is involved in gene and transposon silencing by regulating DNA and histone methylation at heterochromatic loci. DDM1 could remove DNA linker histone H1 at heterochromatic region, allowing DNA methyltransferases access to this area (Zemach et al., 2013). *Arabidopsis ddm1* mutants show a decrease in DNA methylation at heterochromatin regions, which is associated with increasing level of H3K4me2 and a reduced level of H3K9me2 (Tariq et al., 2003). The *KRYPTONITE* gene encodes a histone methyltransferase that is involved in maintenance of DNA methylation. *KRYPTONITE* mutations cause reduced level of H3K9me2, loss of DNA methylation, and reduction in gene

silencing at specific regions (Tariq et al., 2003). In some cases, loss of CG methylation in a *met1* mutant at heterochromatin region are also associated with the decrease of H3K9me2 (Soppe et al., 2002). In *Arabidopsis*, DNA methylation also known to have genome-wide antagonistic relation with histone H2A.Z occupancy. Genomic regions that hyper-methylated is known to be quantitatively deficient in H2A.Z occupancy. Mutation in the DNA methyltransferase *MET1* caused higher H2A.Z incorporation, while mutation in *PIE1 s*ubunit of the Swr1 complex that deposits H2A.Z induced genome-wide hypermethylation (Coleman-Derr and Zilberman, 2012; Zilberman et al., 2008). Besides those direct relations, it is also known that regions in the genome that are repressed usually contain high levels of DNA methylation and low level of histone acetylation (Saze et al., 2012).

### 1.5 Epigenetic Reprogramming During Plants Gametogenesis

In plants, male and female gametes are produced from the differentiation of somatic precursor cell called Pollen Mother Cells (PMCs) and Megaspore Mother Cells (MMCs). Not much is known about the epigenetic state and reprogramming events taking place in PMCs and MMCs. Genome-wide epigenetic data in PMCs and MMCs is not yet available due to the difficulties of isolating sufficient amounts of pure PMCs and MMCs. Plant gametes undergo genome-wide DNA methylation reprogramming during gametogenesis, as occurs in mammals. However, this has been correlated with a large-scale DNA demethylation occurring at the gametes companion cells: the vegetative cells in pollen and the central cell in the embryo sac (Calarco et al., 2012; Hsieh et al., 2009; Slotkin et al., 2009)

In flowering plants the pollen grain is composed of two cells. The first cell is known as the vegetative cell, formed of a large cell surrounded by a thick and strong cell wall. This cell encloses the second cell of the pollen grain, known as the generative cell. In mature pollen, the generative cell will divide to produce two sperm cells. Several studies indicate that the two sperm cells do not undergo significant reprogramming of DNA methylation at CG sites, but methylation at CHH sites is sharply reduced. Sperms cells still have a high level of CG methylation as it is found in somatic cells, especially within the Transposable Element (TE) regions (Calarco et al., 2012; Ibarra et al., 2012; Slotkin et al., 2009). Transcripts from genes that are involved in CG methylation maintenance such as DDM1 and MET1 are highly enriched at sperm cell. Interestingly, the expression of genes that involved in *de novo* DNA methylation such as DCL3 and DRM2 could not be detected in sperm cells (Borges et al., 2008). These findings imply that sperm cell have limited capacity for *de novo* DNA methylation, which might result in the loss of methylation at CHH sites.

In vegetative cells there is massive demethylation at CG sites, accompanied by up-regulation of TE expression and mobility (Slotkin et al., 2009). Unlike CG methylation, the CHG and CHH methylation in vegetative cell is not affected and it is comparable to levels present in somatic cells (Calarco et al., 2012; Ibarra et al., 2012). The expression levels of genes involved in DNA methylation maintenance, such as DDM1 and MET1, are low in vegetative cells while some genes involved in *de novo* methylation such DRM2 and RDR6 are found to be highly active (Jullien et al., 2008; Pina et al., 2005; Slotkin et al., 2009). The DNA glycosylase DEMETER (DME) known to actively remove DNA methylation is also active in the vegetative cell (Schoft et al., 2011). The down-regulation of MET1 and DDM1, and the

expression of DME in vegetative cells have been linked to the reactivation of some TEs. Along with the reactivation of TEs, an increase of smRNAs production has been found in vegetative cells, suggesting that TEs reactivation might induce smRNAs production that could travel to adjacent sperm cells to reinforce TE silencing (Law and Jacobsen, 2010; Slotkin et al., 2009) (Figure 2). This idea was supported by the presence of 21nt siRNAs from Athila retrotransposons found in sperm cells, which are silenced in sperm cells but activate in vegetative cells (Slotkin et al., 2009). This finding supports the model that smRNAs generated in vegetative cells may be transported to sperm cells to silence transposons (Figure 2). However, there is still no direct evidence for smRNAs movement between vegetative cell and sperm cell. A recent experiment showed that artificial microRNAs (amiRNAs) specifically expressed at vegetative cell were unable to establish DNA methylation and silencing at target sequence in the sperm cell (Grant-Downton et al., 2013).

The epigenetic status of female gametes is not yet known. However, through indirect evidence from expression analysis of TEs and DNA methyltransferases, the mechanisms must differ from male gametogenesis as there is a decrease in DNA methylation in both female gametes, egg cell and central cell (Choi et al., 2002; Gehring et al., 2009; Gehring et al., 2006; Jullien et al., 2008). The expression of MET1 and CMT3 is barely detectable both in the egg cell and central cell, suggesting that symmetrical methylation is reduced in those cells. Decreases in DNA methylation are notable in the central cell, leading to activation of several genes and TEs, which are normally repressed in somatic cells (Jullien et al., 2012). As found in the vegetative cell, DNA demethylation in the central cell occurs through the up-regulation of DME, however in the egg cell DME seems to be silenced. DNA demethylation in the central cell is necessary for regulation of maternal imprinting

after fertilization in endosperm (Gehring et al., 2009; Wuest et al., 2010). In contrast to sperm cell, DRM1 and the ARGONAUTE protein family seem to be expressed in the egg cell (Wuest et al., 2010), suggesting that *de novo* methylation is retained in the egg cell but not in the sperm cell. As observed in the vegetative cell, reactivation of TEs may lead to the increase of smRNA production, and as suggested for pollen, and smRNAs produced in the central cell might be transported to egg cell to reinforce TEs silencing (Figure 2). An example for smRNA movement during gametogenesis is shown by the study of Arabidopsis *ago9* mutant lines. AGO9 is a member of Argonaute protein family that is involved in transposon silencing. It has been reported that AGO9-dependent smRNAs silencing plays a crucial role in determining cell fate in *Arabidopsis* ovules. Mutations in AGO9 cause the reactivation of retrotransposons and abnormalities in the megaspore mother cell development. Interestingly, AGO9 is not expressed in reproductive cells, but it is expressed in somatic cells adjacent to reproductive cells (Olmedo-Monfil et al., 2010). Another example is AGO5, which is expressed in companion cells adjacent to reproductive cells during megasporogenesis and regulates initiation of megagametogenesis (Tucker et al., 2012). However, the precise role of these AGO-associated smRNAs produced in companion cells remains unclear.

### 1.6 Epigenetic Reprogramming During Plants Embryogenesis

During sexual reproduction most of the epigenetic changes take place in the endosperm. In *Arabidopsis*, DME is expressed at high-level in the central cell, causing de-methylation and reactivation of some TEs. Following fertilization, DME is active and plays an important role in the global de-methylation process in the endosperm, leading to the activation of some TEs and the production of smRNAs

involved in non-CG *de novo* DNA methylation (Gehring et al., 2009; Hsieh et al., 2009). On the other hand, plant embryos have high levels of DNA methylation primarily at non-CG sites in TEs (Jullien et al., 2012). It has been reported that *dme* mutants has lower levels of DNA methylation of non-CG sites in their embryo compared to the wild-type (Hsieh et al., 2009). There is a possibility that smRNAs produced in the endosperm could regulate non-cell autonomously DNA methylation at embryo (Figure 2). Mosher et al. (2009) reported accumulation of RNA polymerase IV-dependent smRNAs during endosperm development. Whether these smRNAs play a role in communication between endosperm and embryo remains unclear since no RNA polymerase IV-dependent smRNAs were detected in the embryo (Mosher et al., 2009). Nevertheless, DNA methylation plays a crucial part during embryogenesis since *met1* mutations display abnormalities in embryonic cell division and *met1/cmt3* mutants are embryonic lethal (Xiao et al., 2006).

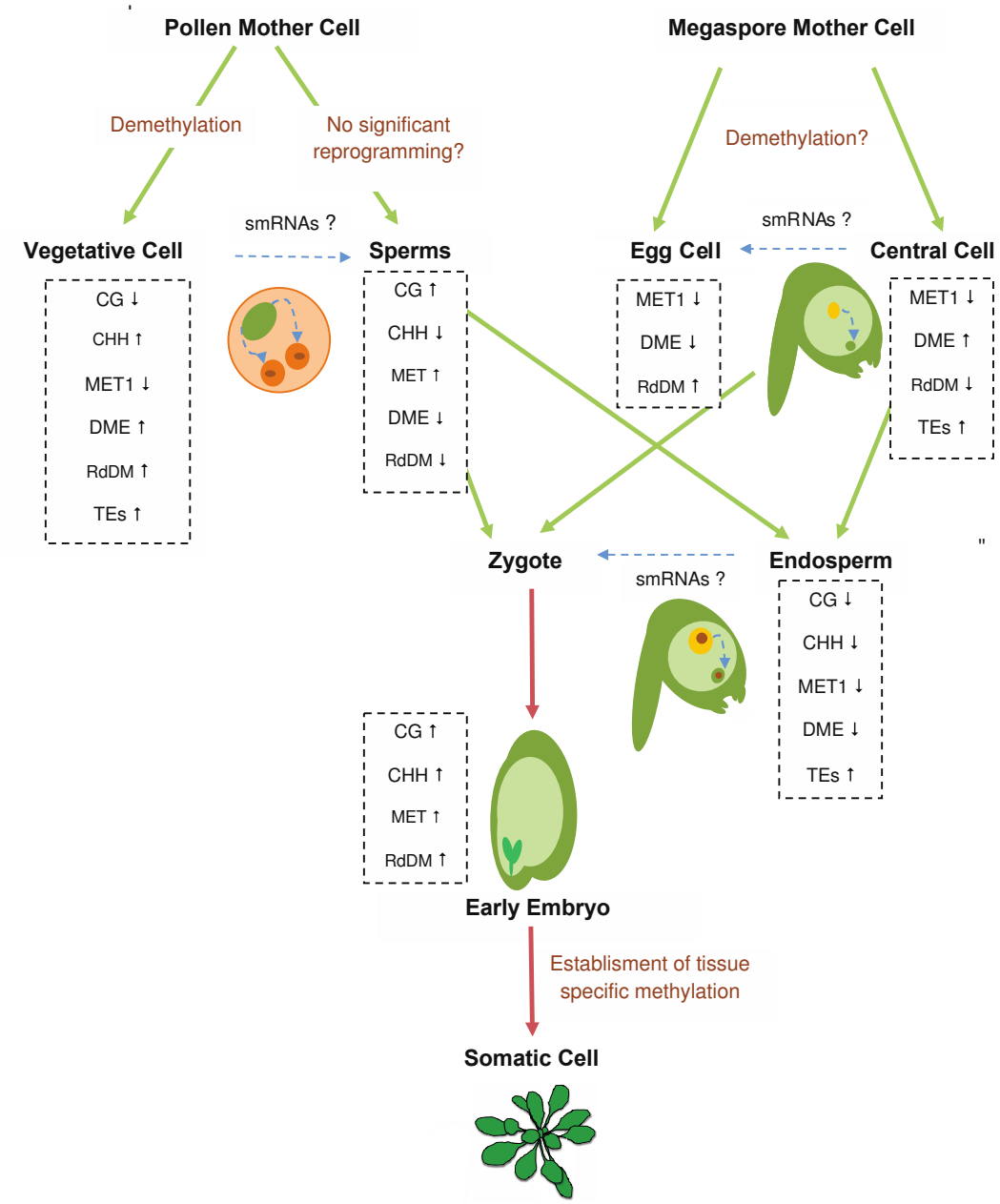


**Figure 2. Epigenetic reprogramming during plants development.** Large-scale demethylation occurs in the vegetative cell causing reactivation of transposons and siRNAs. These siRNA might travel to the sperm cells to establish transposon silencing in the sperms. Massive decrease of DNA methylation level has been proposed to occur in the central cell, leading to activation of TEs and siRNAs. These siRNA may travel to the egg cell to reinforce silencing in the egg cell. Fertilization in *Arabidopsis* produces triploid endosperm and diploid embryo. The endosperm is hypomethylated because of demethylation in the central cell by DNA glycosylase DEMETER (DME). Methylation in all sequence contexts is gradually re-established in the embryo, leading to the formation of tissue specific methylation.

### 1.7 Epigenetic Changes Induced by Environmental Stresses in Plants

The plant epigenome is responsive to environmental stressses and stimuli. Changes in DNA methylation and histone modification occur rapidly following exposure to stress, thus modifying gene expression (Kinoshita and Seki, 2014; Mirouze and Paszkowski, 2011). One of the best known environment-induced epigenetic changes in plants is vernalization, which involves the transcription factor FLOWERING LOCUS C (FLC), a key regulator of floral transition. FLC is highly expressed during *Arabidopsis* vegetative growth and functions as a flowering repressor. After prolonged exposure to cold, FLC expression is repressed by the accumulation of H3K27 tri-methylation accros the entire FLC locus. When normal temperature is restored, H3K27 trimethylation at FLC is retained and flowering is induced (Crevillen and Dean, 2011). FLC expression cannot be found in male or female gametes, indicating that H3K27 trimethylation is not erased during epigenetic reprogramming in gametes. However, after fertilization H3K27 trimethylation is erased by an unknown mechanism so FLC is reactivated in embryo but not in endosperm (Choi et al., 2009; Sheldon et al., 2008). The reactivation process of FLC after fertilization is known to involve exchanges between histone H2A to its isoform histone H2A.Z. This suggests the involvement of histone remodelling in epigenetic reprogramming after fertilization. Besides its role in vernalization response, H2A.Z also involved in developmental response to temperature. H2A.Z nucleosome occupancy changes with temperature, its decreases following increasing temperature to regulate transcriptional response by altering DNA accessibility (Choi et al., 2009; Choi et al., 2007; Kumar and Wigge, 2010). Nevertheless, the mechanism for H2A.Z incorporation during fertilization and its exact role during embryogenesis remains to be elucidated.

Environmental stress could activate components of the epigenetic machinery to establish repressive methylation marks at specific regions and inhibit transcription. In the other hand, it also could promote the release of silencing and activate transcription. Dowen et al. (2012) showed that exposure to biotic stress (*Pseudomonas syringae* pv. tomato DC3000 infection) or salicylic acid treatment caused epigenetic modification at a specific genomic region (TE) and methylation context (CHH but not CG and CHG methylation). Most changes were targeted to TEs, causing hypomethylation and activation of TEs, that could affect transcription of nearby genes. Further analysis showed that methylation changes occured primarily at regions enriched for plant defence regulators and transcription factors (Dowen et al., 2012). Epigenetic regulation in response to stresses however, is not always targeted and fine-tuned. Severe and prolonged environmental stresses could cause genome-wide methylation changes and chromatin instabilities, causing activation of many silenced TEs and genes, independent of their functions. For example, Pecinka et al. (2010) reported that long heat stress treatment causes the activation of some TEs, reduction in nucleosome occupancy and massive dissociation of heterochromatin. By contrast short heat stress had no obvious effect, except for activation of some heat-shock proteins (Pecinka et al., 2010).

Stress-induced epigenetic modifications are often observed at TEs related sequences. While TEs can have mutagenic and deleterious effects through insertions into genes, they may also contribute positively to regulation of plant responses to stress. In rice activation of TE named mPING was associated with cold-responsive expression of nearby genes (Naito et al., 2009), while in *Arabidopsis* activation of TE named ONSEN was associated with heat-stress responsive expression (Ito et al., 2011). Recently, Makarevitch *et al.* (2015) postulated that following abiotic stress treatment

a small number of maize TEs families could regulate the response of adjacent genes by providing stress-responsive enhancer-like functions. Insertion of TEs into protein-coding genes might provide a binding sites for transcription factors or influence chromatin packaging, thus providing regulatory variation in gene expression.

### 1.8 Transgenerational Inheritance of Stress-induced Epigenetic Marks in Plants.

Over the last few years a number of studies have reported in plants the acquisition of new and heritable traits directed by stress. These heritable traits have been attributed to persistent changes in epigenetic marks. The inherited epigenetic marks were observed in promoter regions (Bilichak et al., 2012; Luna et al., 2012), gene-coding regions (Bilichak et al., 2012; Jiang et al., 2014), transgene (Lang-Mladek et al., 2010; Molinier et al., 2006), and especially TEs (Boyko et al., 2010; Dowen et al., 2012; Ito et al., 2011). However, in most cases novel epigenetic marks and acquired traits appear transiently and so far no robust evidence for transgenerational inheritance has been provided (Pecinka and Mittelsten Scheid, 2012). Nevertheless, several studies still propose that epigenetic inheritance in the form of acquired heritable epigenetic marks and stress tolerance may be part of adaptive processes in plants (Kinoshita and Seki, 2014; Mirouze and Paszkowski, 2011) (Figure 3). These studies indicate that stress-induced epigenetic changes that occur in the parental genome can be transmitted to the future generations, hence avoiding epigenetic reprogramming in gametes and embryos. However, the full extent of this reprogramming is still unclear, leaving an open question on how much stress related epigenetic information is transmitted to offspring. Moreover, it is not known if the

epigenetic changes induced by stress are purely stochastic or targeted. In plants, the inheritance of stress-induced epigenetic marks appears to target TEs and intergenic regions but it is unclear how the epigenetic machinery can selectively recognize these genomic regions. It is likely that the repetitive nature of TEs and some intergenic sequences might play a role in recognising these sequences. Several studies have shown that stress responses are impaired in mutants defective in RdDM and in the biogenesis of siRNAs (Boyko et al., 2010; Ito et al., 2011; Luna et al., 2012; Rasmann et al., 2012). These reports support the hypothesis that stress-mediated epigenetic inheritance in plants could rely on the dynamic DNA methylation changes at transposon sequence.

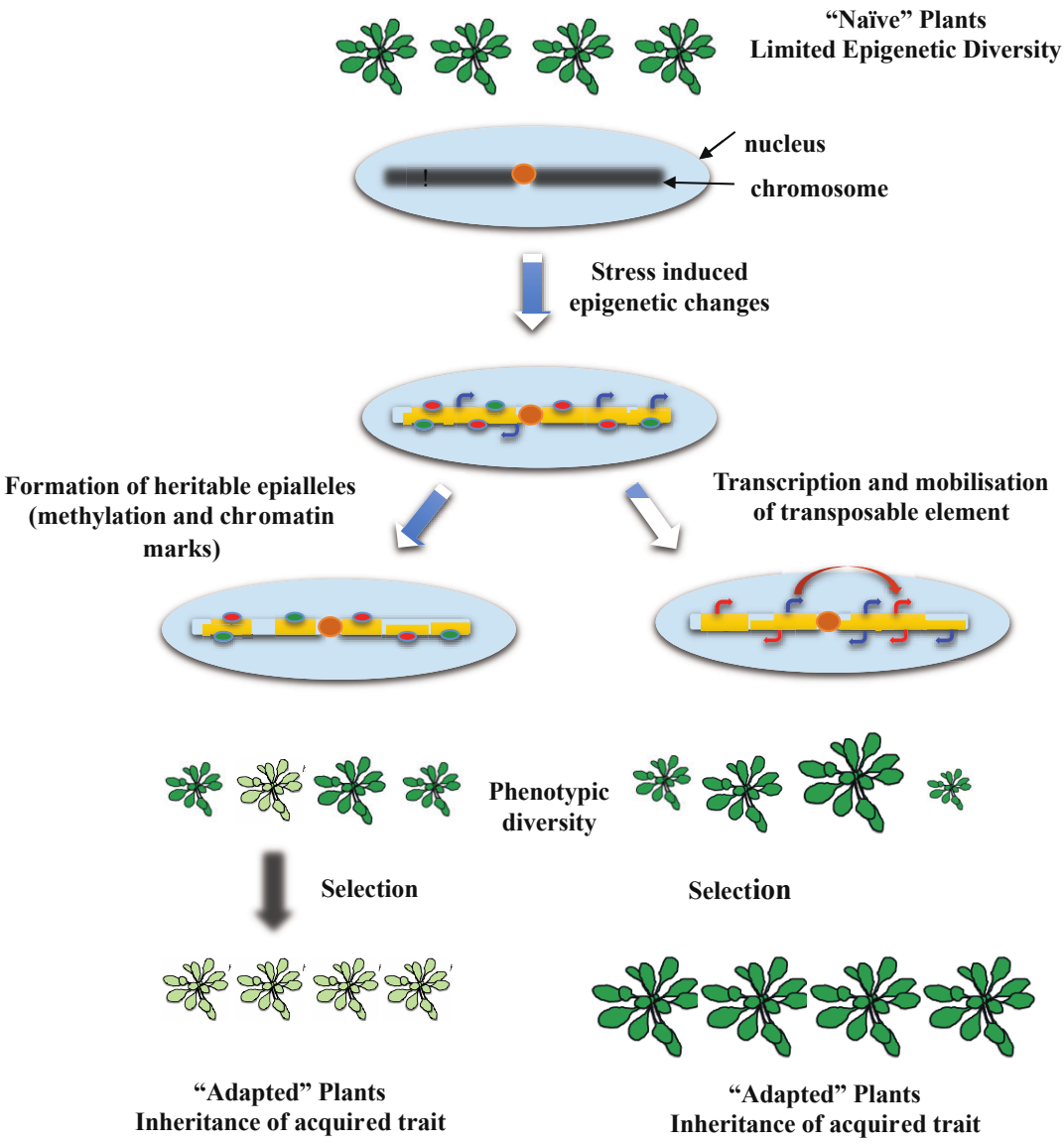


**Figure 3. Proposed model for transgenerational epigenetic inheritance in plants.** Epigenetic regulation is destabilized in plants by environmental stresses causing some epigenetic changes in the genome. Stress induced epigenetic changes can lead to the formation of heritable epialleles (left) and/or activation of transposable elements (TEs) also resulting in their mobilisation. Alteration of epigenetic states could lead to variation in gene expression that could generate phenotypic changes. Some of these phenotypic changes could be stably inherited by the progeny, providing the progeny with new adaptive advantage.

## 1.9 Aims and Hypothesis

The extent and mechanism by which organisms acquire epigenetic changes and heritable adaptive traits after exposure to environmental stress is a central question in genetics and evolution. Plants present a good model to address such questions due to their sessile nature and well-studied epigenetic landscape. Several studies have reported that environmental stress could induce genome-wide DNA methylation changes and may provide adaptive benefits to the progeny (Bilichak et al., 2012; Dowen et al., 2012; Jiang et al., 2014). However, the current studies have not been able to fully explain the extent and stability of stress-induced epigenetic changes, their mode of inheritance, or their adaptive value to the progeny.

The aims of this project is to address those three fundamental questions by performing a robust and systematic DNA methylation analysis of plant populations exposed to salt stress for five consecutive generations followed by non-stress exposure for a further two generations. In addition, I am also assessing the dynamics of DNA methylation in plants' gametes following salt stress to determine how methylation marks are inherited to the offspring. Further, I would like to evaluate the roles of stress-induced epigenetic changes in phenotypic and transcriptional response to salt stress.

Several studies have suggested that DNA methylation in plants is dynamic and responsive to stress and that stress-induced methylation changes are required for efficient respond to environmental changes (Boyko et al., 2010; Dowen et al., 2012; Kinoshita and Seki, 2014). However it is strongly debated whether stress-induced methylation changes is stable and heritable. I hypothesize that multigenerational salt stress treatments could lead to novel epigenetic changes and adaptive traits that stably inherited across non-stressed generations. I also hypothesize that stress-induced epigenetic changes are not occurred stochastically but targeted to certain stress-responsive genic regions or TEs adjacent to genes. These targeted methylation changes might alter the transcriptional response of stress related genes and

providing plants with novel adaptive/phenotypic response to stress. It has been previously proposed that during gametogenesis, the plant male germline undergoes extensive DNA methylation reprogramming (Calarco et al., 2012). Several studies have suggested that the inheritance of stress memory in plants may be regulated under maternal control under maternal control (Agrawal, 2001; Pecinka and Mittelsten Scheid, 2012). In accordance to these, I hypothesize that salt-induced methylation marks and adaptive traits will be inherited maternally due to the active resetting of methylation marks in the male gametes.

# 2. Materials and Methods

## 2.1 Plant Material

For multi-generation salt stress treatment *Arabidopsis thaliana* reporter line L5 which harbours a marker gene encoding L-glucuronidase (GUS) linked to the 35S promoter (35Spro::GUS) was used, this line was obtained from Dr. Ortrun Mittelsten-Scheid (Gregor Mendel Institute). In Col-0 wild-type background, the promoter driving the expression of the GUS reporter is methylated and silenced. Silencing of the *GUS* transgene is transiently released following heat and salt stress treatments (Pecinka et al., 2010). To facilitate isolation of the male gametes, a specific reporter line was used. This line carried the promoter of the MALE-GAMETE-SPECIFIC HISTONE H3 (MGH3) gene fused to eGFP (*pMGH3::MGH3-eGFP*) and the promoter of ACT11 gene fused to a chimeric histone H2B protein fused to mRFP (*pACT11::H2B-mRFP*). These two reporters were crossed to produce a double homozygous plant harbouring both markers (Appendix Figure 28) (Borges et al., 2012) and seeds were provided by Dr. Jörg Becker (The Instituto Gulbenkian de Ciência) .

To evaluate molecular mechanism that regulates inheritance of epigenetic marks in response to salt stress, six epigenetic mutant lines were also subjected to multi-generation salt stress treatment. The epigenetic mutant lines being used and their description are shown in Table 1.

**Table 1**. List of mutant lines being used in this study.

| Name | Source | Description |
|---|---|---|
| *nrpda1-4* (Herr et al., 2005) | David Baulcombe (Cambridge University) | NRPD1A encodes one of two of largest subunit of RNA polymerase IV. It is required for the synthesis of 24-nt siRNAs which involved in *de novo* DNA methylation |
| *cmt3-11* (Chan et al., 2006) | David Baulcombe (Cambridge University) | CMT3 encodes chromomethylase involved in CHG methylation and preferentially methylating transposable element related sequence. |
| *drm1-2/drm2-2* (Chan et al., 2006) | David Baulcombe (Cambridge University) | DRM1 and DRM2 double mutant line. Both of the genes encode methyltransferase required for *de novo* CHH methylation |
| *ddc* (Chan et al., 2006) | David Baulcombe (Cambridge University) | Triple mutant of DRM1, DRM2, and CMT3 |
| *ros1-4* (Penterman et al., 2007) | David Baulcombe (Cambridge University) | ROS1 encodes DNA N-glycosylase required for DNA demethylation. It is function as a repressor of transcriptional gene silencing. |

| | | |
|---|---|---|
| *dme-6* (Shirzadi et al., 2011) | Claudia Köhler<br><br>(Swedish University of Agricultural Science) | DME encodes DNA N-glycosylase required for DNA demethylation expressed at high-level in the companion cells of plant gametes |
| *rdd* (Penterman et al., 2007) | Daniel Zilberman<br><br>(University of California Berkeley ) | Triple mutant of ROS1, DML2, and DML3. All of those genes encode proteins with DNA N-glycosylase activity that is involved in DNA demethylation. |

### 2.2 Plant Growth Conditions

For all experiments *Arabidopsis* seeds were vernalized by incubation at 4°C for 48 hours in dark. Seeds were germinated and grown in a growth chamber (Conviron) or glasshouse under following regime: 16 h day, 8 h night photoperiod, at 22 °C, light intensity 120 µmol/sec/$m^2$. Seeds for plate-grown seedling were surface sterilized by shaking on 10% of Sodium hypochlorite (VWR) for 10 minutes, then washed in sterile $H_20$ for 5 times. All seeds were germinated and grown on Murashige and Skoog (MS) salts (Duchefa Biochemie) with 0.7% phytoagar (Duchefa Biochemie) and 1% sucrose (Sigma-Aldrich).

### 2.3 *Arabidopsis* Crosses

*Arabidopsis* crosses were performed by hand on inflorescences from 4-6 weeks old plants. Sepals and petals were carefully removed using fine forceps to expose the

anthers. The anthers were then removed carefully (by emasculation) without touching the stigma or pistil. The emasculated stigma then leaved for two days. After 2 days crosses was carried out by rubbing a suitable anther from a mature flower onto the emasculated stigma. Successful crosses were marked after elongation of pistil was apparent.

### 2.4 Multigenerational Salt Treatments

Seeds from single *Arabidopsis* L5 plants (S0) were used for multigenerational salt treatment. S0 seeds were germinated and grown on MS media (control) or MS media with 25 or 75 mM NaCl (treatments) for two weeks. Two weeks old seedling were then transferred accordingly to phytatray (Sigma-Aldrich) containing 125 mL of MS media (control) or MS media with 25 or 75 mM NaCl. After 4 weeks in phytatray all the plants were transferred to normal soil without salt, these plants were named S1. For each group of treatments, ten S1 plants were used. Leaf samples and seeds were collected separately from each S1 plant. This process was repeated for five successive generations. In each generation, offspring of the salt treated and control plants were grown in non-stress condition (soil) for two successive generations to produce P1 and P2 plants.

### 2.5 Salt Tolerance Assay

#### 2.5.1 Germination and Survival Test

For germination assay, six pools of 50 seeds were germinated on MS media supplemented with 200 mM NaCl for 14 days. Seeds were scored as germinated based on radicle emergence. For survival assay, six pools of 50 seeds were

germinated and grown on MS media supplemented with 150 mM NaCl or 125 mM KCl or 300 mM Mannitol. The survival rate of *Arabidopsis* seedling was scored after 14 days based on the presence of green leaves.

### 2.5.2 Chlorophyll Content Assay

*Arabidopsis* plants were grown on MS media supplemented with 100 mM NaCl for 5 weeks. Leaves from control and salt-treated plants were collected, weighted fresh, and washed in distilled water. Chlorophyll were extracted by incubating 0.02 – 0.03 gr of grinded leaf in 80% (v/v) acetone (Sigma-Aldrich) at 4°C for 48 hours in the dark. After 48 hours the sample was diluted 4 times using 80% acetone (250µL of samples mixed with 750 µL of 80% acetone). Chlorophyll content was measured using a spectrophotometer at 663.6 nm and 646.6 nm absorbance. Total chlorophyll content (chlorophyll a and b) was calculated using following equation:

Chlorophyll **a** (µg/ml) = 12.25 ($A_{663.6}$) – 2.55 ($A_{646.6}$)

Chlorophyll **b** (µg/ml) = 20.31 ($A_{646.6}$) – 4.91 ($A_{663.6}$)

Total chlorophyll (µg/ml) = 17.76 ($A_{646.6}$) + 7.34 ($A_{663.6}$)

### 2.5.3 Sodium Content Assay

*Arabidopsis* plants were grown on MS media supplemented with 100 mM NaCl for 5 weeks. Leaves from control and salt-treated plants were collected and washed in distilled water. Plants materials were dried on 80°C for 48 hours. After 48 hours 0.01 gr – 0.1 gr of dried samples was transferred into 50-ml polypropylene and 2 ml of concentrated nitric acid was added. After being mixed well, the sample tubes were placed inside microwave digester. The digestion program consisted of: 5 minutes on

100° C, 2 minutes on 120° C, 5 minutes on 160° C, 22 minutes on 180° C, and cooling down to 70° C. After samples cooling down, the digested samples were diluted with 23 mL of distilled water. The sodium ions concentration inside the digested samples then measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

### 2.5.4 Dry Weight Measurement

*Arabidopsis* plants were grown on MS media supplemented with 100 mM NaCl for 5 weeks. Aerial parts from five-week-old plants were excised then dried at 80°C for 48 hours to determine dry weight.

### 2.5.5 Root Elongation Assay

*Arabidopsis* plants were grown vertically on MS media for 10 days. The 10 days old seedling were then transferred to MS media supplemented with 175 mM NaCl and the position of root tip was immediately marked after transfer. After two weeks growth on MS media supplemented with salt, the root elongation was quantified by measuring the root elongation using ImageJ..

## 2.6 Isolation of Sperm and Vegetative Cell Nuclei.

### 2.6.1 Collection of Pollen Grains

Pollen samples were collected only from S1 plants. Progenies from single *MGH3::MGH3-eGFP/ACT11::H2B* (S0) were germinated and grown on MS media (control) or MS media with 25 or 75 mM NaCl (treatments) for two weeks. Two

weeks old seedling then transferred to phytatray (Sigma-Aldrich) contain 125 mL of solid MS media with or without NaCl accordingly. After 4 weeks in phytatray the plants were transferred to soil to produce flowers. Mature *Arabidopsis MGH3::MGH3-eGFP/ACT11::H2B* flowers were collected into 50 ml falcon tube, with a volume approximately 10 ml, then 10 ml of sperm nuclei buffer was added (45 mM MgCl2, 30 mM Sodium Citrate, 20 mM MOPS, 1% Triton-100, pH 7.0) and the falcon tube was vortexed vigorously for 3 minutes. The pollen suspension was then filtered through a Miracloth mesh and centrifuged for 1 minute at 3000 rpm, then the supernatant was carefully removed to get pellet of pollen. The pollen grains were stored at -80 °C until required.

### 2.6.2 Extraction of Vegetative Nuclei and Sperm Nuclei

For extraction of vegetative and sperm cell nuclei from intact pollen grains, first the pollen pellet was re-suspended on 1 ml sperm nuclei buffer. The pollen suspension was then loaded into 1.5 ml eppendorf tube containing 100 µl volume of acid-washed glass beads (425 – 600 µm) and subjected to vortexing for 4 minutes. The crude extract was then filtered through 28 µm micro-filter sieve, this method leaves the vegetative and sperm nuclei intact.

### 2.6.3 Purification and Isolation of Vegetative Nuclei or Sperm Nuclei by Fluorescense Activated Cell Sorting (FACS)

The filtered crude extract of disrupted pollen then processed using Fluorescent activated cell sorting was carried out with a MoFlo (Beckman Coulter, Fort Collins, USA) using laser tuned to 488 nm laser at 140 mW used for forward scatter (FSC),

Side Scatter (SSC) measurements and for GFP excitation. Another laser tuned to 561 nm at 38 mW for RFP excitation. GFP and RFP were detected using a 530/40 nm and a 630/75 nm bandpass filters, respectively. To minimalize loss of sperm and vegetative cell, which has average diameter 2.5 μm, the threshold of FSC was set in low (Appendix Figure 28). Phosphate Buffer Saline (PBS 1X) was used as sheath, and run at a constant pressure of 400 kPa (~60 psi). Frequency of droplet formation was approximately 96,000 Hz. The sorted vegetative nuclei and sperm nuclei were collected separately into 1.5 ml eppendorf tube containing 1 ml of sperm nuclei buffer, and stored at -80 °C until required for further analysis.

### 2.7 DNA Extraction

For whole genome sequencing of somatic cells, for each treatment group, rosette leaves were polled from 10 plants. For male gametes analysis, for each treatment group, pollen was collected from 100 plants. Rosette leaves were collected when the plants were 7 weeks old, while pollen was collected when the plants were 7-10 weeks old. Leaf material was flash-frozen in liquid nitrogen and pulverised using mortar. The gDNA extraction from leaf samples was performed using DNAeasy Plant Mini Kit (Qiagen) according to manufacture instruction. The gDNA extraction for sperm and vegetative cells were performed using MasterPure$^{TM}$ DNA Purification Kit (Epicentre).

## 2.8 RNA Extraction

Several genes that showed differential methylation in response to multigenerational salt stress treatment were chosen for qRT-PCR analysis to evaluate the correlation between methylation and gene expression. The P1 and P2 seeds of S3 control and treatment group were grown on MS media supplemented with 125 mM NaCl for 2 weeks. For each treatment group, leaves were collected from 50 seedling, leaf material were flash-frozen in liquid nitrogen and pulverized using mortar. Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen) according to manufacture instruction.

## 2.9 Quantitative Real-Time PCR Analysis

Extracted RNA was treated with TURBO DNA-free$^{TM}$ (Promega, Madison, WI) following the manufacturer's instructions. cDNA was synthesized from 1 µg of extracted RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. All RT-qPCR analyses were performed using a MyiQ System (BIO-RAD) with the MESA Blue qPCR MasterMix Plus reagent (Eurogentec Headquarters). Using Primer3 software (Rozen and Skaletsky, 2000) specific primers were designed for the sequences of selected genes. The list of primers used in qRT-PCR can be found in Appendix Table 4. The PCR fragments were analysed using a dissociation protocol to ensure that each amplicon was a single product. Amplicons were also sequenced to verify the specificities of the targets. The amplification efficiency was calculated from raw data using the LingRegPCR software (Ramakers et al., 2003). All RT-qPCRs were performed using five biological replicates in a final volume of 25 µl containing 5 µl of cDNA template (diluted beforehand 1:10), 0.2 µM of each primer, and 12.5 µl of

2×MESA Blue qPCR MasterMix (Eurogentec Headquarters) according to the manufacturer's instructions. The following thermal cycling profile was used: 95°C for 10 min, followed by 40 cycles of 95° C for 10 s, 60° C for 15 s, and 72° C for 15 s. Following cycling, the melting curve was determined in the range of 60–95° C, with a temperature increment of 0.01° C/sec. Each reaction was run in triplicate (technical replicates). Negative controls included in each run were a reaction conducted in absence of reverse transcriptase and a reaction with no template (2 μL of nuclease-free water instead of 2 μL of cDNA). Raw data from the MyiQ System were exported to a data file and analysed using the GeneEx Pro software (Kubista et al., 2006). Analysis of expression data was performed according to the ddCT method (Livak and Schmittgen, 2001) using AtGAPDH (At1g13440), AtPDF2 (At1g13320) and AtUBIQUITIN5 (At3g62250) as housekeeping genes for normalization (Lippold et al., 2009). To measure CNI1 antisense lncRNA transcripts, 5 □g of total RNA isolated from seedlings was reverse-transcribed into cDNA with SuperScript III, primed by three forward oligonucleotides (Appendix Table 4). The resulting cDNA was used as template in semi- quantitative PCR to amplify the CNI lncRNA with specific primers (Appendix Table 4). PCR reactions were performed in duplicate and RT-minus controls were included to confirm absence of genomic DNA contamination.

### 2.10 Library Preparation for Bisulfite Sequencing

DNA libraries for bisulfite sequencing were generated using the Illumina TruSeq Nano kit (Illumina, CA, USA) according to the manufacturer's instructions. DNA was sheared to 350 bp. The bisulfite treatment step using the Epitect Plus DNA Bisulfite Conversion Kit (Qiagen, Hilden, Germany) was inserted after the adaptor

ligation. After clean-up of the bisulfite conversion reaction, library enrichment was done using the Kapa Hifi Uracil+ DNA polymerase (Kapa Biosystems, MA, USA) according to the manufacturer's instructions. Leaf material from 10 individual plants were pooled, in order to eliminate inter-individual or spontaneous DNA methylation variation.

### 2.11 Sequencing

Bisulfite sequencing was performed on an Illumina HiSeq 2000 instrument. Libraries were sequenced with 2 x 101 bp paired-end reads; with conventional genomic DNA libraries sequenced in control lanes for base calling calibration. Seven to eight libraries with different indexing adapters were pooled in one lane. For image analysis I used Illumina RTA 1.13.48.

### 2.12 Processing and Alignment of Bisulfite-converted Reads

The procedure followed the one previously described in Becker et al. (2011). The SHORE pipeline v0.9.0 (Ossowski et al., 2008) was used to trim and quality-filter the reads. Reads with more than or 5 (or 2) bases in the first 25 (or 12) positions with a base quality score of less than 5 were discarded. Reads were trimmed to the right-most occurrence of two adjacent bases with quality values equal to or greater than 5. Trimmed reads shorter than 40 bases were discarded. Reads were then aligned against the Col-0 reference genome sequence using SHORE (Ossowski et al., 2008).

### 2.13 Determination of Methylated Sites

The process was as described in Becker et al. (2011). The number of covered and methylated sites for each sample as well as the false methylation rates retrieved from read mappings against the chloroplast sequence can be found in Appendix Table 5. On average, 40.7 million cytosines were covered by at least 3 reads and with a quality score above 25 in more than half of the samples. Of these, 7.2 million cytosines were methylated in at least one sample.

### 2.14 Identification of Differentially Methylated Positions (DMPs)

The same methods as in Becker et al. (2011) were applied, with the following difference: sites classified as differentially methylated between replicates were not removed from the analysis. Fisher's exact test were applied on the 7.2 million cytosine sites methylated in at least one samples for all pairwise sample comparisons. The same *P* value correction scheme as in Becker et al. (2011) were used with the difference that I only considered pairwise comparisons where the methylation rate between two samples differed by an absolute value of minimum 30%.

### 2.15 Identification of Methylated Regions (MRs)

MRs was identified using a Hidden Markov Model (HMM), following the same process as in Hagmann et al. (2015). MRs was identified in each sample separately. MRs of replicates were merged into a common set of MRs. Whenever different samples were treated as a replicate group (e.g. control and salt-treated samples, respectively), their MRs were merged into a common set (Hagmann et al., 2015).

### 2.16 Identification of Differentially Methylated Regions (DMRs)

The method followed the one previously described in Hagmann et al. (2015). Regions that showed statistically significant methylation differences between at least two sets of strains were identified as DMRs. In brief, segmentations of the genomes of every sample served to set breakpoints of start and end coordinates of all predicted MRs. Each combination of coordinates in this set defined a segment to perform the test for differential methylation in all pairwise comparisons of the strains, if at least one strain was in a high methylation state throughout this whole segment. Per pairwise comparison, between 30,000 and 50,000 segments were tested. Testing regions for differential methylation, grouping differentially methylated samples for each region, and testing regions between groups of samples were done according to Hagmann et al. (2015). For test within generations, I grouped P0 control, P1 control and P2 control samples as “non-stressed”; P0 salt-treated samples as “stressed”; P1 samples derived from salt-treated P0 plants as “stressed-P1”; and P2 samples derived from salt-treated P0 plants as “stressed-P2”. Tests for DMRs were then performed between these four groups. In addition, I did separate tests without the respective remaining groups for “non-stressed” vs. “stressed”, “stressed” vs. “stressed P1”, “stressed” vs. “stressed P2”, and “stressed P1” vs “stressed P2”. This latter step was done to assess the number of DMRs directly identified between two groups without multiple testing corrections taking into account comparisons with and between other groups.

### 2.17 Mapping to Genomic Elements

TAIR10 annotation for genes, exons, introns and untranslated regions; transposon annotation was done according to (Slotte et al., 2013). Positions and regions were hierarchically assigned to annotated elements in the order CDS > intron > 5' UTR > 3' UTR > transposon > intergenic space. I defined intergenic positions and regions as those that were not annotated as either CDS, intron, UTR or transposon. Each position was assigned to the corresponding element that contained it. DMRs were assigned to annotated elements by basepair, i.e. each position in the DMR was assigned in the above-mentioned order. By this, a DMR can stretch over several annotated elements.

### 2.18 Overlapping Region Analysis

I tested the overlap of DMRs with other DMRs or with genes using bedtools (Quinlan and Hall, 2010) either requesting a direct overlap or an overlap within a window of n basepairs downstream and upstream of the regions. The overlap between DMRs with Transposable Elements (TEs), histone marks, and long non-coding RNA (lncRNA) were tested using custom Perl scripts, either requesting a direct overlap or an overlap within 2000 basepairs windows downstream and upstream of the DMRs. For DMRs overlap with TEs, overlapping TEs were then sorted into their super families according to TAIR10 nomenclature. These TEs profiles for hypo and hyper methylated DMRs were then compared against the expected values taken from the whole genome TEs profile.

### 2.19 Gene Ontology Analysis

I used Protein ANalysis THrough Evolutionary Relationships (PANTHER) software (Mi et al., 2013) to classify significantly enriched Gene Ontology (GO) terms associated with Mutation Accumulation (MA) Overlap/Nooverlap and Hypo/Hypermethylated DMRs. Heatmaps for GO analysis were generated using R version 3.0.1 (www.r-project.org).

### 2.20. Statistical analysis

Student's unpaired t-test was performed using GenStat. Hierarchical clustering was done in R version 3.0.1, using the heatmap.2 function of the gplots package in combination with the hclust function of the fastcluster package (www.r-project.org).

### 2.21 Data Visualization

Graphical displays were generated using R version 3.0.1 (www.r-project.org). Circular display of genomic information in was rendered using Circos version 0.63 (Krzywinski et al., 2009a, b)

### 2.22 Data Accessibility

The DNA and RNA sequencing data have been deposited at the European Nucleotide Archive under accession numbers PRJEB9076. DNA methylation data and MR coordinates have been uploaded to the epigenome browser of the EPIC Consortium

## 3. The Impact of Multigenerational Salt Exposure on *Arabidopsis thaliana* Stress Adaptation.

## 3.1 Introduction

### 3.1.1 Intra-generational Response to Stress in Plants

Environmental stress has a negative impact on plants growth, survival and reproduction, therefore plants able adapting to stress have a better chance to survive and reproduce in a fluctuating environments. Adaptation and response to stress involves a complex series of changes in gene transcription, hormones signalling, and biochemical processes. These complex responses are required to minimize stress-induced damage and to repress growth and development during stress exposure. However, once stress is lifted normal development and growth need to be restored. Therefore, plants stress responses must be sensitive and precise, but also reversible (Atkinson and Urwin, 2012).

In general plants responses to stresses are controlled through several interdependent mechanisms. First, extracellular signals are sensed by trans-membrane receptors, inducing transcriptional changes in these cells. The primary receptor triggers signalling cascades involving oxidative, hormone and calcium-dependent signals. These signalling cascades result in the activation of stress-responsive gene networks, which can be common between stresses or specific to particular stress. (Deinlein et al., 2014).

Plants usually respond to stress via short-term reversible mechanism. Depending on the type, duration and intensity of the signal, stress can also induce long-tem adaptive responses such as: acclimatization (reversible adjustment of cellular process and physiological regulation for days or weeks within plants lifetime), irreversible modification of plants structure, or epigenetic and genetic adaptation across several generations (Roy et al., 2012). However, most molecular and physiological

responses directed by stress are short-lived. For example, drought stress induces Abscisic Acid (ABA) accumulation in stomatal guard cells, affecting calcium and potassium efflux from the cell, and causing rapid stomatal closure. ABA effect on stomatal behaviour is short-term (hours to days) and does not alter stomata functions permanently (Kim et al., 2010). Heat stress can also induce short-term tolerance in plants. Heat stress induces activation of Heat-Shock Protein (HSP) through changes in protein structure and HSPs are required to repair miss-folded or denaturated proteins. In *Oryza sativa* production of HSP comes to end less than 3 hours after stress exposure (Goswami et al., 2010). However, a repeated exposure to heat stress could induce plant acclimatization to heat. It has been described that repeated heat stress leads to a higher level of HSP and increased level of superoxide dismutases isoenzymes that last for several days, making the plants more efficient at photosynthesis and resistant to subsequent heat stress (Camejo et al., 2007).

In addition to heat stress, other environmental stresses are known to induce acclimatization. Sani et al. (2013) reported that mild salt stress treatment in *Arabidopsis* seedling resulted in adult plant that displayed reduced salt uptake and enhanced drought tolerance (Sani et al., 2013). Plants exposure to low non-freezing temperature are also known to cause increased tolerance against subsequent freezing temperature. Initial cold exposure activates the expression of cold response pathway, including genes that stabilize cells membrane and protects proteins against severe dehydration that occurs with freezing, providing plants with better protection against subsequent cold exposure (Thomashow, 1999).

Adaptive responses to stress are not always reversible. For example, severe drought stress exposure can induce high level of ABA production, which could alter plant's morphology. ABA promotes root growth and inhibits shoot development during

drought stress, resulting in significant increase of root to shoot ratios (Sharp and LeNoble, 2002). Bigger and longer roots allow plants to penetrate deeper into soil to collect more water. In addition, inhibition of shoot growth reduces the number and surface size of leaves, thus minimalize the water lost through evaporation. Plants exposed to repeated drought also develop a thicker cuticle layer in the epidermis of leaves, thus reducing water loss (Kosma et al., 2009). Moreover, drought-stressed plants produce leaves with smaller stomata but with higher stomatal density in the lower epidermis. These modifications in plants morphology and structures are not reversible and will give plants better tolerance against repeated drought exposures (Xu and Zhou, 2008). Beside drought stress, it has been reported that continuous attack by pathogens or herbivores could lead to production of leaves with higher density of trichome. Higher density of trichome act as a physical barrier, restricting pathogen and herbivore access to leaf surface, thus providing resistance to attacks (Tian et al., 2012).

### 3.1.2 Inter-generational Response to Stress in Plants

Exposure to stress can result in changes that are transmitted across multiple generations. A number of recent studies in plants have reported that plants exposed to environmental changes can perceive and "memorize" stress. This "stress memory" could be passed to future generation and influenced phenotypes of the progeny (Kinoshita and Seki, 2014; Mirouze and Paszkowski, 2011). In *Arabidopsis*, stress has been shown to induce genetic instability that can be passed to the next generation. Molinier et al. (2006) reported that ultraviolet-C or flagellin treatment could increase homologous recombination rate of a β-glucuronidase (*GUS*) transgenic reporter and that increased level of homologous recombination is

transmitted to the non-stressed progeny. This genetic instability is associated with changes in the epigenetic state of treated plants that could affect the somatic recombination machinery (Molinier et al., 2006). Kovalchuk et al. (2003) also reported that tobacco plants infected with either tobacco mosaic virus (TMV) or oilseed rape mosaic virus (ORMV) displayed increased homologous recombination of a transgenic luciferase reporter (Kovalchuk et al., 2003). Further, Boyko et al. (2007) reported that the progeny of TMV-infected tobacco displayed increased instability and hypomethylation at a Leucine-Rich Repeat (LRR) gene cluster, which are involved in resistance to pathogen attack (Boyko et al., 2007). Another study using a 35S:GUS transgene reporter has showed that Transcriptional Gene Silencing (TGS) is released following heat stress (42 °C), cold stress (-4° C) and ultraviolet-B treatment. This stress-induced TGS release is retained in the next two non-stressed generations, but only for a small number of cells in leaves (Lang-Mladek et al., 2010). In a separate study, Pecinka et al. (2010) also reported that various physical and chemical stresses (including heat stress and ultraviolet-B) could induce increased level of homologous recombination and reduced TGS of a reporter gene. However, they also showed that the next two non-stressed progenies only showed low frequency and stochastic homologous recombination. There was not correlation between the level of stress stimulation given to the parental plants and recombination rate. Therefore, the authors then concluded that a stress memory is not a general response to stress in plants (Pecinka et al., 2010).

Further evidence for transgenerational response to abiotic stress comes from studies on exposure to high salinity. Boyko et al. (2010) reported increased global DNA methylation and higher levels of tolerance against salt stress in the direct progenies of salt-stressed plants. However, the observed acquired tolerance appear to be lost in

the successive non-stressed generations. They also showed that the stress tolerance obtained depended on the action of Dicer-like (DCL) 2 and DCL3, which encode dicer proteins involved in RNA-directed DNA methylation (RdDM) (Boyko et al., 2010). It has been also shown that salt-stress induces hypermethylation at certain regions of the *Arabidopsis* genome (Bilichak et al., 2012).

Exposure to high temperature during reproductive stages also affects the phenotype of progenies. Progenies of *Arabidopsis* grown under warm conditions (25°C) displayed faster germination, root elongation, and higher biomass when compared to progenies of plants grown in cold temperature (15°C) or grown under normal condition (20°C). However, after exposure to freezing temperature (-5°C) plant progenies displayed better photosynthesis efficiency that correlated with the up-regulation of several cold-responsive genes (Blodner et al., 2007). In separate study, Whittle (2009) reported that parental exposure to heat stress could also improve the fitness of the progeny (Whittle, 2009).

Biotic stress treatments are also associated with the induction of transgenerational adaptive responses. Acquired tolerance following biotic stress was reported to be stably inherited across many non-stressed generations. Luna et al., (2012) reported that progenies of *Arabidopsis* infected with *Pseudomonas syringae* pv *tomato* DC3000 (PstDC3000) were more resistant to biotrophic pathogen *Hyaloperonospora arabidopsidis*. This improved resistance was retained over three successive generations without stress (Luna et al., 2012). Tomato and *Arabidopsis* exposed to caterpillar herbivory, methyl jasmonate and mechanical damage also showed similar transgenerational effects. Caterpillar grows up to 50% smaller in progenies of stressed-plants and this adaptive response was retained for two successive non-stress generations (Rasmann et al., 2012). Not only pathogen or herbivory attack were

efficient in directing transgenerational stress response, treatment with β-amino-butyric acid (BABA) also induced priming responses that resulted in an enhanced resitance to biotrophic pathogens (Slaughter et al., 2012). The transcriptional changes induced by pathogen attack were found to be associated with two chromatin marks: an enrichment of acetylated H3K9 at SA-inducible genes and an enhancement of H3K27me3 at JA-inducible genes. Acetylation of H3K9 is correlated with transcriptional activation, while enrichment of H3K27me3 is associated with transcriptional silencing. Similar to salt stress, trangenerational response to biotic stress is affected in RNA directed DNA methylation (RdDM) mutants (Agrawal, 2001; Luna et al., 2012; Luna and Ton, 2012; Slaughter et al., 2012). In summary, parental exposure to both biotic and abiotic stress can affect the phenotype of the progeny. In some cases the newly acquired phenotypes could persist without stress over several generations. Collectively, these data indicate that stress memory responses in plants are regulated epigenetically.

### 3.1.3 Chapter Aims

The aim of this chapter is to evaluate on *Arabidopsis* the effect of multi-generational salt stress treatment in adaptation to high salinity.

## 3.2. Results

### 3.2.1 Mutigenerational Salt Stress Treatments in *Arabidopsis*.

Biotic and abiotic stresses have been implicated in transgenerational responses where non-stress progenies displayed increased tolerance to stress (Kinoshita and Seki, 2014; Mirouze and Paszkowski, 2011; Paszkowski and Grossniklaus, 2011). Previous studies have investigated transgenerational responses induced by short exposure to high salinity. However, in nature plants could constantly be exposed to different type stresses over many generations. Therefore, repeated exposure to salt stress over several generations may enhance transgenerational adaptive responses in plants. To evaluate the effect of multi-generation salt stress treatments on plant adaptation to high salinity, I grew *Arabidopsis* plants for five consecutive generations in media containing two different salt concentrations (25 and 75 mM NaCl). To examine the stability of stress induced epigenetic and phenotypic changes, the offspring of the salt treated plants were grown in non-stress condition for two successive generations (Figure 4A).

In this experiment, I have chosen to apply salt stress treatments that cause growth suppression and delay in flowering but did not affect plant survival or sexual reproduction. Plants grown on control condition start bolting after 4 to 5 weeks, while salt stressed plants bolting after 6 to 8 weeks (Figure 4B). To prevent a direct effect of the salt treatment on the developing seeds, plants were moved to salt-free medium (soil) after 6 weeks, just before the plants start flowering. The salt treatment was given using culture media to make sure that all the plants equally exposed to same level of salt concentration. Application of salt to plants grown on soil pots could lead to unequal accumulation of salt in the soil over time.

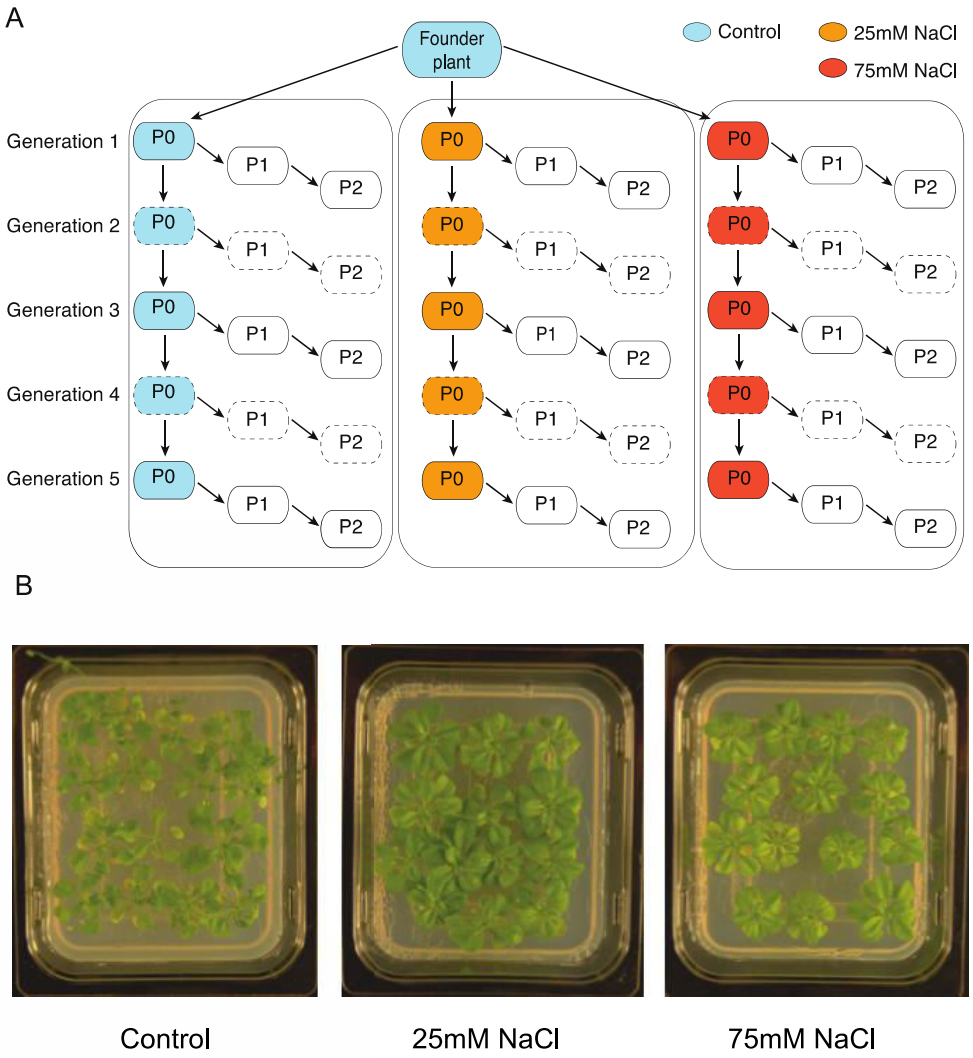


**Figure 4. Schematic diagram of the salt stress treatment and the effect of salt stress treatment on *A.thaliana* development.** (A) Seeds from a single *A. thaliana* L5 plant (Founder plant) were used for the multigenerational salt treatment. Plants were grown for 6 weeks on control or salt-containing medium (25 mM and 75 mM NaCl, respectively), before being transferred to soil for maturation. Salt stress treatment was applied for five constitutive generations. From each generation, offspring of the salt treated and control plants were grown in non-stress condition for two successive generations to produce P1 and P2 plants. Plants from generation 1, 3 and 5, and of their respective P1 and P2 progeny were used for bisulfite sequencing (solid-lined boxes), while plants from generation 2 and 4 were not used for for bisulfite sequencing (dotted-lined boxes). (B) Growth repression and delayed flowering in 6 weeks salt stressed plants.

### 3.2.2 Repeated Exposure to Salt Stress Leads to Transient Adaptation to High Salinity in the Progeny

To evaluate the extent of the transgenerational responses following multigenerational salt stress treatment, I grew seeds from control and salt treated plants on media with high concentration of salt. There is no different in germination and survival rate between the progeny of control and salt treated plants when they were germinated and grown in MS media without salt. The progeny of control and salt treated plants, both showed 92-100% germination and survival rate in MS media without salt (Appendix Figure 26). However, in media with high salinity the first progeny (P1) of plants that have been exposed to 25 mM NaCl or 75 mM NaCl displayed higher survival and germination rates compared to the progeny of untreated control plants (Figure 5 and Figure 6A). Only 45-59 % of the control plants survived and formed green leaves two weeks after sowing on 150 mM NaCl, while progeny of salt-stressed plants showed 68-80% survival rate. Similarly, only 59-68% of the control seeds germinated and developed roots on 200 mM NaCl, while seeds from salt-treated plants germinated with an efficiency of 77-96%. Moreover, when plants were grown on 100 mM NaCl for 5 weeks, chlorophyll content was reduced in control plants when compared to the progeny of salt-stressed plants (Figure 6). These results indicate that the progeny of stressed plants can acquire an increased tolerance to high salinity when plants are exposed over multiple generations.

Enhanced tolerance to salinity is often associated with the ability to exclude sodium from tissues, better growth of root system and increased plant biomass under high-salinity. However with the exception of P1 plants of generation 2, descendants of salt-stressed plants did not show changes in the accumulation of sodium in leaves (Appendix Figure 27). The progeny of control and salt treated plants also did not

show a significant different in root elongation rate or aerial biomass (dry weight) when grown on media with high salinity (Appendix Figure 27).

Interestingly, the phenotypic differences between progenies of control and salt-treated plants became apparent only after the parents had experienced salt stress treatment for two or more generations, and were not detectable if progenies of plants that had been subjected to salt stress only for one generation. These results suggest that two consecutive salt treatments are necessary to trigger increased tolerance in the P1 (Figure 5 and Figure 6). It also suggests that a single exposure to salt is not sufficient to trigger an intergenerational response, and that multigenerational salt treatments are required to induce heritable changes.

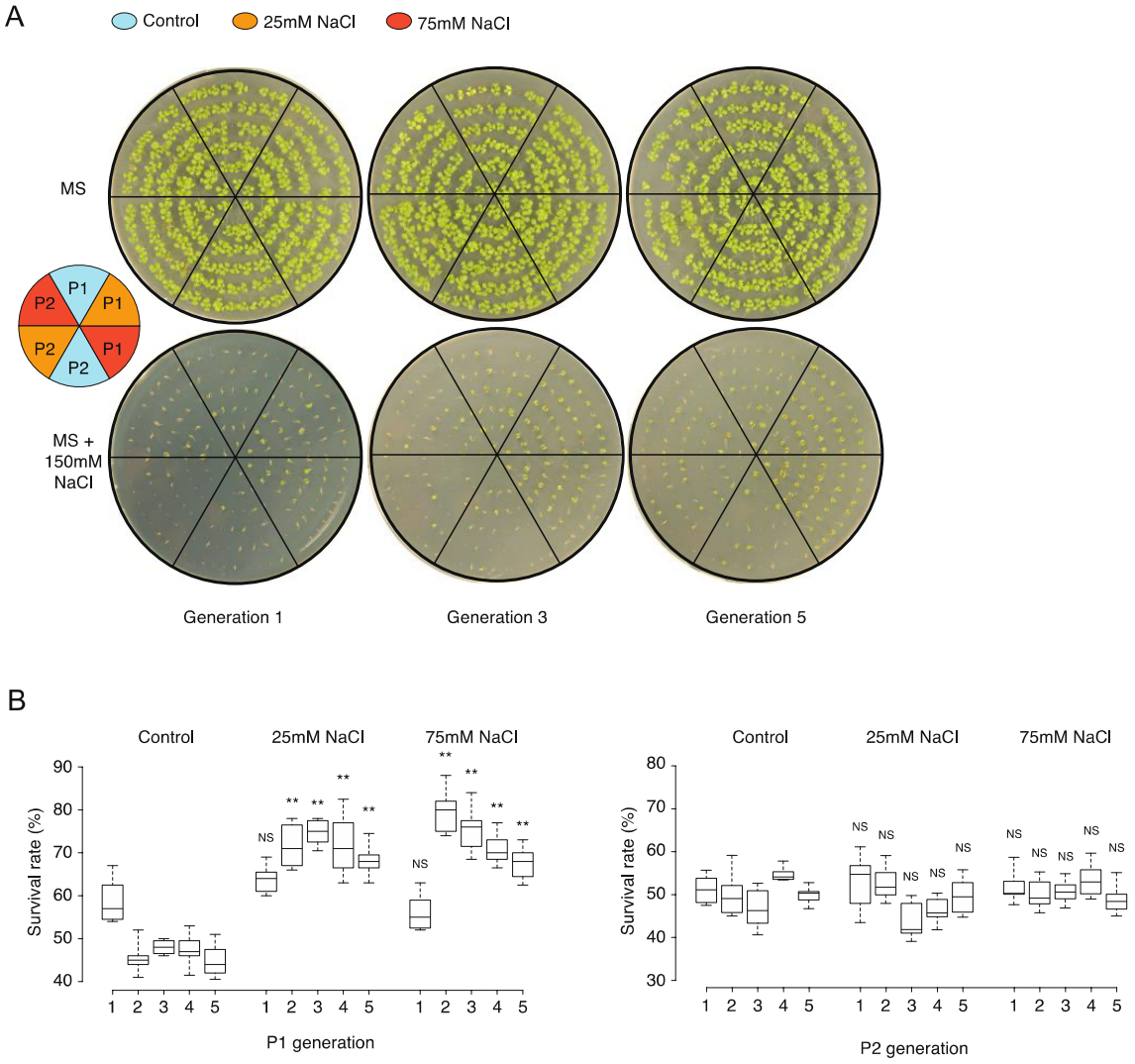


**Figure 5. Limited inheritance of acquired tolerance following multigenerational salt stress treatment.** (A) Salt tolerance assay of the P1 and P2 progeny of control and salt-treated plants in generations 1, 3 and 5. Seeds were germinated on MS or MS + 150 mM NaCl; pictures were taken 2 weeks after sowing (B) Survival rates of P1 and P2 seedlings grown on salt-supplemented medium. For each sample and treatment I analysed 6 plates and assessed 50 seedlings per plate. Asterisks indicate a significant difference to the control of the same generation (unpaired Student's *t*-test; ** $p<0.01$; * $p<0.05$; NS $p>0.05$).

To assess the heritability of the phenotypes acquired by P1 progenies, I grew P1 plants from all generations under salt-free conditions to produce P2 plants. Surprisingly, none of the P2 displayed enhanced survival or germination rates when compared to control plants nor did I detect an elevated chlorophyll content (Figure 5 and Figure 6). Taken together these results suggest that recurrent salt stress treatment in plants induces intergenerational adaptation that allows the direct progeny to respond better to salt stress. However, these responses do not persist in subsequent generations in the absence of stress. Collectively, these results suggest that plants have developed mechanisms to encode and erase information induced by environmental stress.

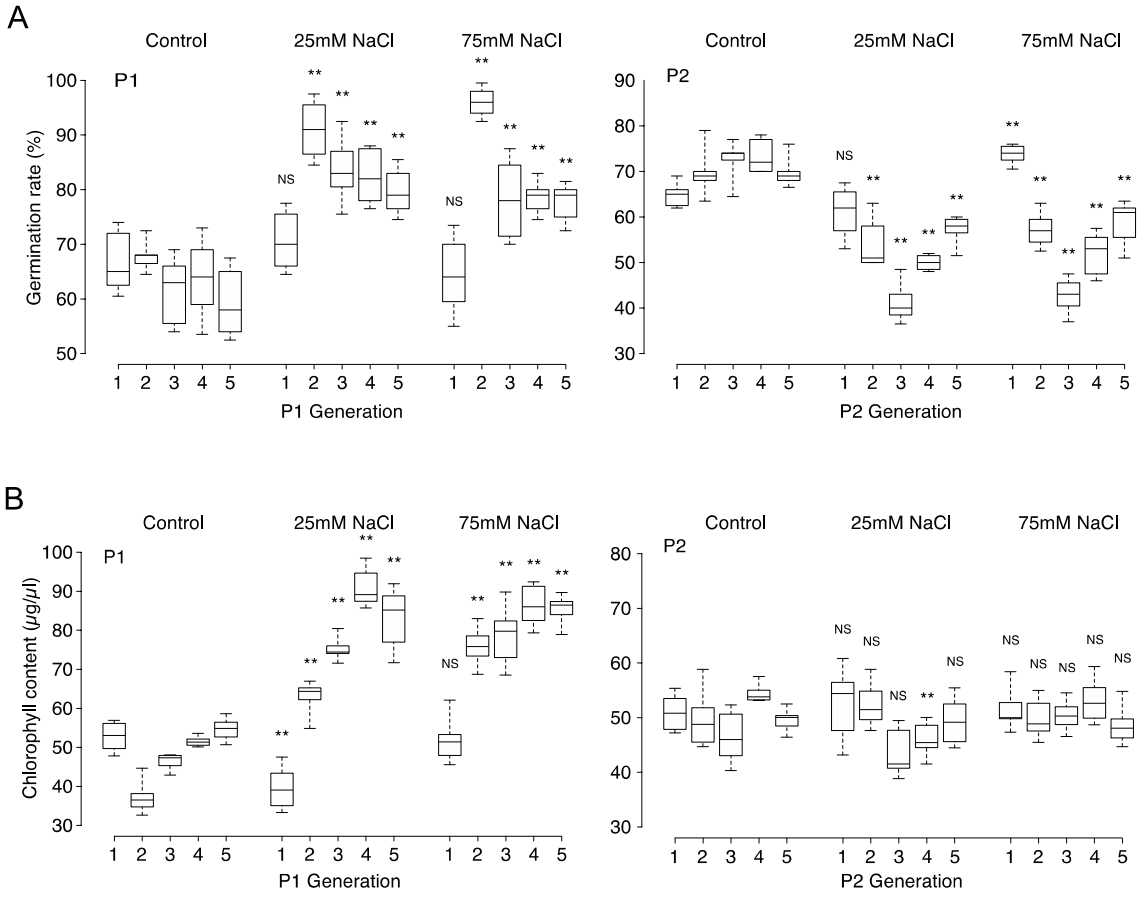


**Figure 6. Salt tolerance assays for P1 and P2 progeny of control and salt-treated plants** (A) Germination rates of P1 and P2 seeds on medium supplemented with 200 mM NaCl. For each sample and treatment I analysed 6 plates and assessed 50 seedlings per plate. (b) Chlorophyll content of P1 and P2 plants grown for 5 weeks on medium supplemented with 100 mM NaCl (For each treatment group I analysed 6 plants). (Asterisks indicate a significant difference to the control of the same generation (unpaired Student's *t*-test; ** $p<0.01$; * $p<0.05$; NS $p>0.05$).

### 3.2.3 First progeny (P1) of Salt-stressed Plants is Osmotolerant

Salt stress treatment with sodium chloride causes cellular osmotic and ionic stress. My data shows that exposure t*o* mild NaCl stress for five constitutive generations resulted in higher germination rate, survival rate, and chlorophyll content in the first progeny. Therefore, I decided to evaluate whether the acquired tolerance in P1 is specific for NaCl or also found for other salts or osmotic agents, KCl and Mannitol respectively. I found that the P1 plants displayed enhanced tolerance to NaCl, KCl and Mannitol stress. However, the acquired tolerance to these stress agents in the P1 was absent in P2 progenies (Figure 7).

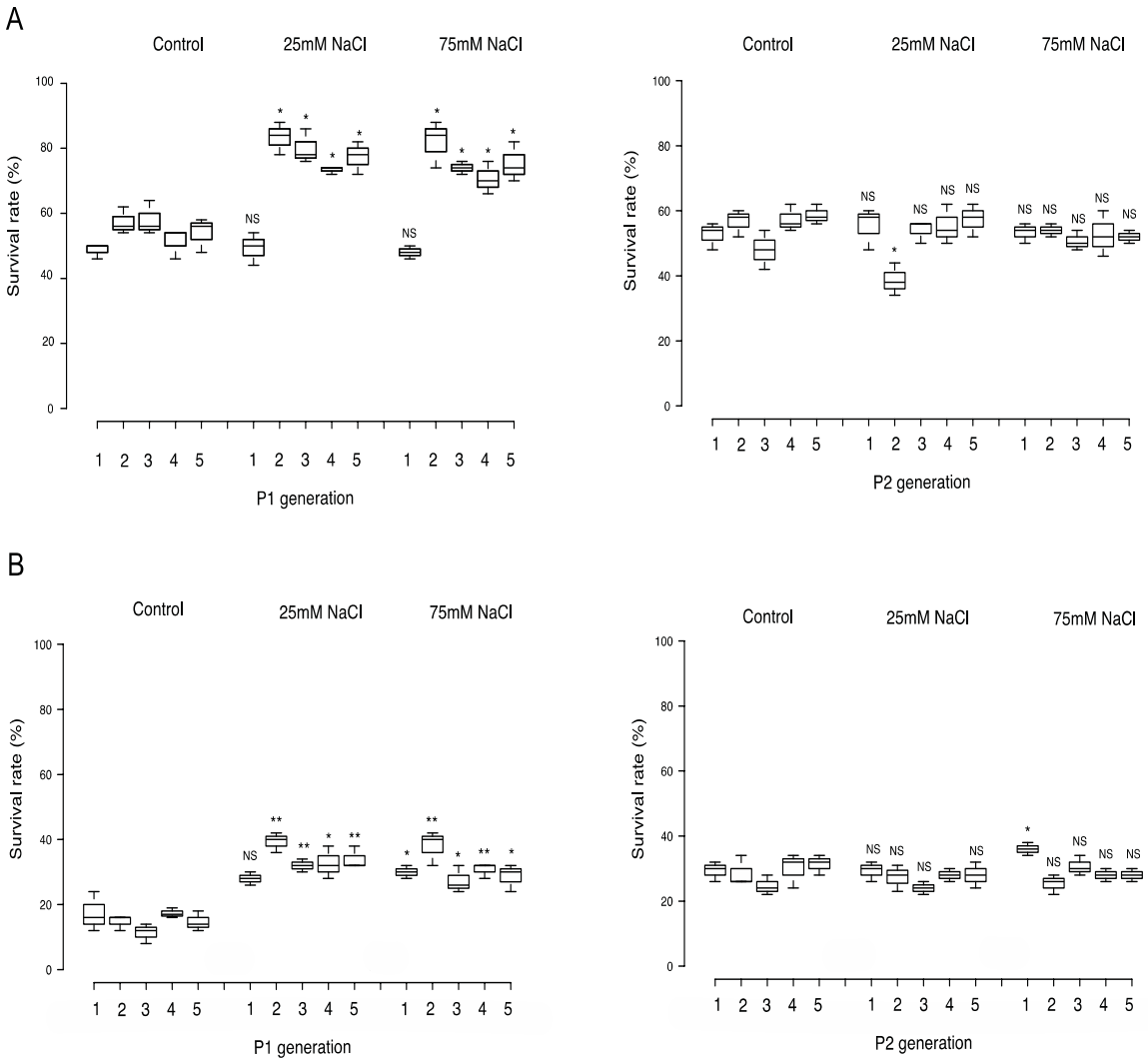


**Figure 7. P1 progeny of salt-treated plants exhibit increased tolerance to Mannitol and KCl.** (A) Survival rates of P1 and P2 seeds on medium supplemented with 300 mM Mannitol. For each sample and treatment I analysed 6 plates and assessed 50 seedlings per plate. (B) Survival rates of P1 and P2 seeds on medium supplemented with 125 mM KCl. Asterisks indicate a significant difference to the control of the same generation (unpaired Student's *t*-test; ** p<0.01; * p<0.05; NS p>0.05).

### 3.2.4 Adaptation to Salt Stress is Impaired in DNA Methylation Mutants

The large number of individuals analysed in my study and the loss of phenotype within one generation indicates that the acquired stress tolerance observed in the progeny of plants exposed to salt is not caused by genetic changes. Several studies suggest that environmental stress induces genome-wide epigenetic changes that can be transmitted to the offspring (Bilichak et al., 2012; Dowen et al., 2012; Jiang et al., 2014). To evaluate whether the enhanced tolerance seen in the offspring of stressed plants were due to newly acquired epigenetic changes, I subjected various *Arabidopsis* mutants that are affected in RNA- directed DNA methylation (RdDM) or in the active removal of DNA methylation (Law and Jacobsen, 2010; Zhang and Zhu, 2012) to salt stress for two successive generations, and tested their progenies for tolerance of high-salinity.

For this analysis I used mutants in NRPD1A- one of the two large subunits of RNA polymerase IV that it is required for the synthesis of RdDM-related 24-nt siRNA (Herr et al., 2005), DRM1 and DRM2 double mutant line, methyltransferases required for *de novo* CHH methylation (Chan et al., 2006), a mutant in the DNA methytransferase CMT3 –mainly involved in the regulation of Transposable Element (TE) CHG methylation (Chan et al., 2006), a triple mutant of DRM1, DRM2 and CMT3 (Chan et al., 2006), a single mutant of ROS 1 and triple mutant of ROS1, DML2 and DML3, DNA glycosylases that mediate DNA demethylation (Penterman et al., 2007). There is no different in survival rate of the control and salt treated plants, in wild-type or mutant background, when the plants were grown in MS media without salt (Figure 8A). However when the plants were grown in media with high salinity, unlike the wild-type plants, none of the epigenetic mutants showed enhanced tolerance to salt stress in the P1 (Figure 8B). Intriguingly, contrary to WT

salt exposure in *cmt3* and *nrpd1a* produced P1 progenies that were less tolerant to high salinity. The tolerance level of in *cmt3* and *nrpd1a* were reverted back to normal level in the non-stressed P2 progenies (Figure 8C). Together these data imply that transgenerational adaptation to salt stress required DNA methylation machinery.

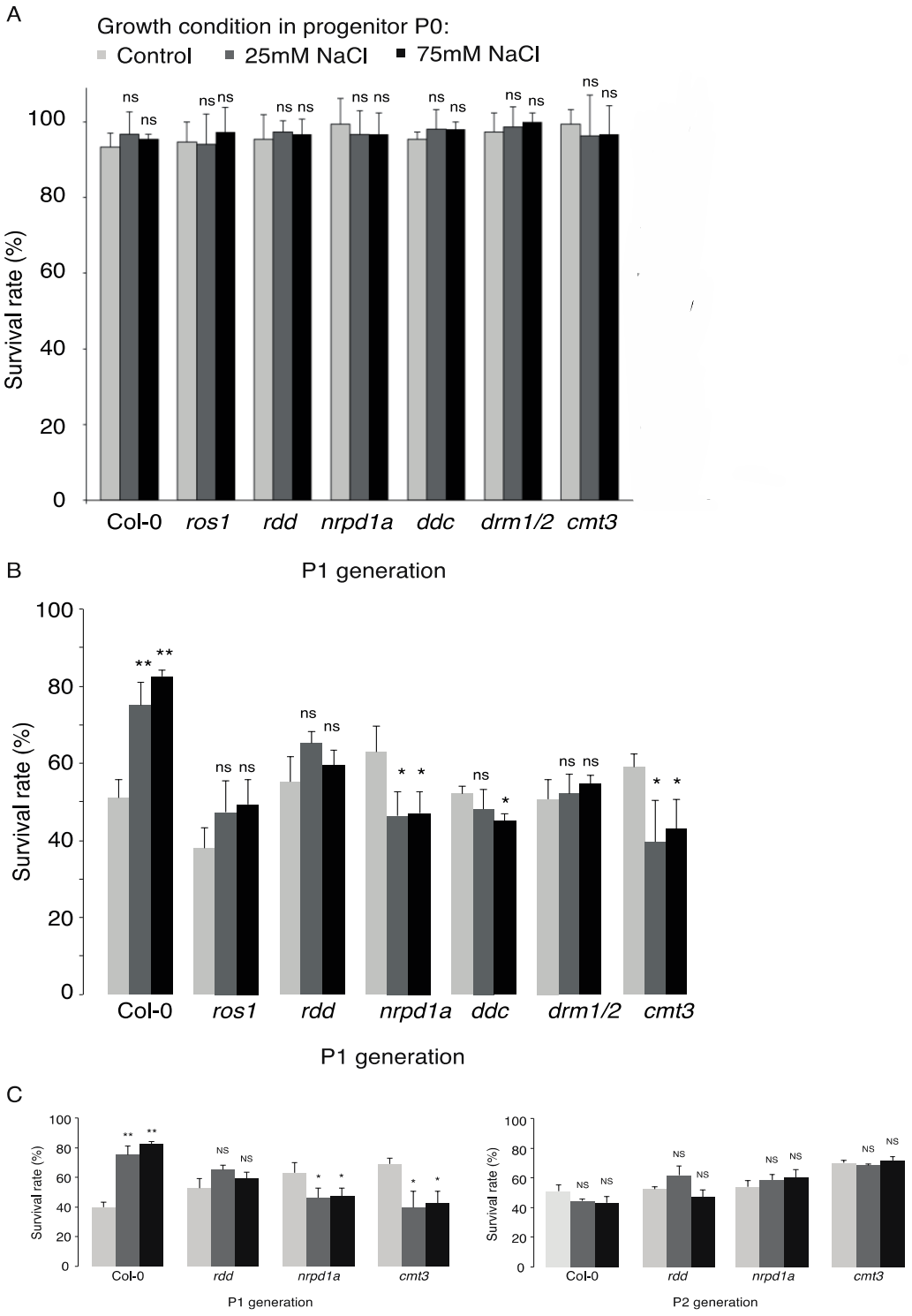


**Figure 8. DNA methylation mutants were not able to adapt to salt stress.** (A) Survival rate of P1 progeny from wild-type and various epigenetic mutant lines on MS medium without salt and (B) MS medium supplemented with 150M NaCl. (C) Survival rate of P1 and P2 progeny from wild-type and three epigenetic mutant lines on 150M NaCl. Survival rates are shown in percentage (average of three plates, calculated from 50 seeds per plate). Asterisks indicate significant difference to the respective control (unpaired Student's *t*-test; ** p<0.01; * p<0.05; NS p>0.05) (ddc: *drm1; drm2; cmt3* triple mutans, rdd: *ros1; dml1; dml2* triple mutant).

### 3.2 Discussion

Previous studies reported that exposure to salt stress could induce transgenerational response in form of improved tolerance and increased methylation rate in the progenies (Bilichak et al., 2012; Boyko et al., 2010). In this study I found that progenies of salt-stressed plants displayed better germination, survival and chlorophyll content in high salinity compared to progenies of control plants (Figure 5 and 6). The adaptive phenotypes observed in P1 were apparent only after two consecutive exposures to high salinity (Figure 5 and Figure 6) thus suggesting that transgenerational adaptive stress responses require repeated stress stimuli. Sani et al. (2013) also found that plants exposed to salt during early stages of development displayed enhanced tolerance to high salinity during adulthood and that this effect was associated with altered chromatin and transcriptional responses. Plants that were exposed to salt only once during the adult stage did not displayed salt-resistant phenotypes (Sani et al., 2013). Boyko et al. (2010) on the other hand reported that a single salt stress treatment was sufficient to induce tolerance in the direct offspring (Boyko et al., 2010). The discrepancies revealed in these studies may be due to differences in timing, duration and strength of the stress, and differences in growth conditions. Moreover, these experiments were carried out using two different *Arabidopsis* ecotypes that might differ in their adaptive behaviour to high salinity (Katori et al., 2010). Boyko et al observed an enhanced tolerance in the progeny of Arabidopsis C24 plants in response to 75 mM NaCl for 3 weeks from germination. While in this study I exposed Arabidopsis Col-0 plants to 25 or 75 mM NaCl for 6 weeks from germination. Previous study reported that Arabidopsis ecotype Col-0 and C24 displayed varied transcriptional regulation of salt responsive genes in response to salt stress. These different in transcript level correlate with variation in

tolerance level between the two ecotypes, where C24 showed better tolerance to salt stress compared to Col-0 (Jha et al., 2010). Given the considerable variation in NaCl tolerance between Arabidopsis ecotypes, it is likely that transgenerational adaptation to salt stress will also differ between different ecotypes.

Because exposure to sodium chloride causes both osmotic and ionic stress (Deinlein et al., 2014), I assessed if P1 plants responded differently to stress mediated by potassium chloride or mannitol. I found no significant differences between P1 survival rates after exposure to NaCl, KCl and Mannitol, thus suggesting that P1 plants acquired a general tolerance to osmotic and ionic stress (Figure 7).

Because several studies have suggested that stress induced tolerance can be inherited over multiple generations in the absence of stress (Kinoshita and Seki, 2014; Mirouze and Paszkowski, 2011), I grew control and salt-stressed P1 plants under salt-free conditions. I found that even after five constitutive generations of salt stress, the second-generation grown under non-stress conditions (P2) did not show any noticeable adaptive phenotypes on high salinity. These results indicate that the enhanced tolerance to high salinity was largely reset in the absence of stress, thus suggesting plants have developed mechanism(s) to acquire and erase information acquired during stress. This reversible adaptive response may be critical for plants as they are sessile and could enable them to cope with recurrent environmental fluctuations.

Because the heritability of stress-induced tolerance has been primarily associated with changes in DNA methylation (Kinoshita and Seki, 2014; Mirouze and Paszkowski, 2011), I exposed several epigenetic mutants to salt stress for two successive generations and tested their progenies for tolerance to high-salinity. This

analysis revealed that unlike Col-0 mutants defective in DNA methylation and demethylation pathway showed no improved tolerance in the P1 (Figure 8). Interestingly, following salt stress for two generations *cmt3* and *nrpd1a* produced progenies that were less tolerant to high salinity (Figure 8). CMT3 is the main regulator of CHG methylation at transposons, which concurs with previous reports indicating that stress affects the epigenetic state of TE located in euchromatic regions of the genome (Dowen et al., 2012; Makarevitch et al., 2015). Changes in the epigenetic state of TEs are often accompanied by an increase in small non-coding RNA production (smRNAs) (Saze et al., 2012) and the synthesis of TE-derived smRNAs is mediated by NRPD1A, one of two of largest subunit of RNA polymerase IV (Herr et al., 2005). Moreover, this smRNAs are required to direct *de novo* CHH and CHG methylation at discrete sequences of the genome (Matzke and Mosher, 2014).

### 3.4 Summary

In summary, my data supports the view that plants can integrate environmental signals and generate offspring better adapted to stress. However, in subsequent generations in the absence of stress this adaptive response is gradually lost. My data also imply that adaptation to salt stress relies on the DNA methylation machinery,

# 4. DNA Methylation Changes Upon Multigenerational Salt Stress Treatment and Their Mode of Inheritance

## 4.1 Introduction

### 4.1.1 DNA Methylation Changes in Response to Stress

DNA methylation is a dynamic and reversible regulatory system that could serves as adaptive mechanism to various environmental stimuli. Several studies have reported that environmental stress could induce genome-wide DNA methylation changes. The loss or gain of methylation in response to stress could lead to activation or repression of stress-responsive genes and providing plants with better adaptation to stresses (Kinoshita and Seki, 2014; Mirouze and Paszkowski, 2011). One of earliest evidence for the occurrence of stress induced DNA methylation was provided by Steward et al. (2002). They reported that cold stress treatment (4 °C) on maize seedling caused genome-wide demethylation in root tissue. A putative protein and retrotransposon-like sequence called ZmMI1 was identified as one of the targets for demethylation. Interestingly, ZmMI1 is only transcribed during cold stress, suggesting that ZmMI1 demethylation is required for its activation (Steward et al., 2002). Further work from the same group identified set of genes that differentially expressed in transgenic tobacco plants expressing an anti-sense construct of DNA methyltransferase NtMET1. One of the genes they identified, NtAlix1, was differentially expressed and methylated in response to tobacco mosaic virus (TMV) infection, suggesting that pathogen response in tobacco involved the methylation machinery (Wada et al., 2004).

Changes in DNA methylation following salt stress treatment have been also observed in various plant species. In *Arabidopsis thaliana*, salt stress induces global genome hypermethylation. Gain of methylation in response to salt stress were found in Transposable Elements (TEs), gene promoters, 5′ and 3′ ends of gene body, and in

exons rather than introns. In addition, it has been shown that the expression of salt-induced hypermethylated genes was repressed (Bilichak et al., 2012). Global changes in DNA methylation also observed following multi-generational salt stress treatment. Jiang et al. (2014) reported that following salt stress treatment for 10 successive generations lead to the accumulatation of ~45% differentially methylated positions at CG-sites (CG-DMPs). However, this result is surprising since it has been shown previously that salt stress mainly induce changes at non-CG sites in intergenic regions (Bilichak et al., 2012; Boyko et al., 2010). Notably, Bilichak et al (2012) showed that salt induced CG-DMPs modifications at genic regions (CDS, intron, UTR an non-coding RNAs) rather than non-genic regions (intergenic, pseudogene and TEs). In tobacco, salt stress was reported to induce transcription and CG-demethylation at coding regions of glycerophosphodiesterase-like (NtGPDL), thus suggesting a transcription-methylation relationship in response to stress (Choi and Sano, 2007). In maize, salt stress induced hypermethylation of retrotransposons and genes that regulate transcriptional stress responses. These data reveals the importance of DNA methylation dynamics in salt stress responses (Tan, 2010). In asexual apomictic dandelion, salt stress triggers methylation changes throughout the genome. However, the salt-induced methylation changes varied between individual replicates, suggesting the presence of random and variable individual epigenetic responses (Verhoeven and van Gurp, 2012). In rice, a study using four different rice genotypes examined the effect of salt stress in two different tissues (root and shoot) showed that methylation changes are tissue and genotype specific. However, this study could not establish a direct link between DNA methylation and changes in gene expression (Karan et al., 2012). Collectively, these studies suggest a role for DNA methylation in stress responses. It is possible that DNA methylation in plants

is dynamic and responsive to stress and that stress-induced methylation changes are required to induce transcriptional changes and enabling plants to efficiently respond to environmental changes.

### 4.1.2 The role of RdDM Pathway in Stress Response

Most studies mentioned have shown that DNA methylation plays an important role in stress tolerance by regulating the expression of stress responsive genes. In addition, some of them indicate that salt stress could induce wide-scale DNA methylation changes. In some cases, these changes are targeted to specific genome regions. Recognition of specific sequences in the genome in response to specific stress stimuli will required the involvement of particular methylation or demethylation pathway. In fact, RNA Directed DNA methylation (RdDM) has been proposed as key epigenetic stress response pathway (Boyko et al., 2010; Dowen et al., 2012; Ito et al., 2011). RdDM is a major methylation pathway in plants that directed by small RNAs (Matzke and Mosher, 2014). RdDM is initiated by the production of double-stranded RNAs (dsRNAs) through the plant-specific RNA polymerase Polymerase IV and the RNA DEPENDENT RNA POLYMERASE 2 (RDR2). These dsRNAs are processed into 24 nucleotide small interfering RNAs (siRNAs) by DICER-LIKE 3 (DCL3) and then incorporated into the AGO4 protein complex. The siRNAs-AGO4 complex could direct *de novo* DNA methylation at region homologous to the siRNAs in all sequence contexts (CG, CHG and CHH) by interacting with nascent Polymerase V transcript scaffold and DNA methyltransferase DRM2 (Matzke and Mosher, 2014) (Figure 9).

Recently, an alternative RdDM pathway implicating the RNA DEPENDENT RNA POLYMERASE 6 (RDR6) and 21-22-nt siRNAs has been reported in *Arabidopsis* (Mari-Ordonez et al., 2013; Matzke and Mosher, 2014; Nuthikattu et al., 2013). The pathway is initiated by the transcription of heterochromatic transposons by DNA Polymerase II (POLII). Some of these transcripts are amplified by RDR6 to produce dsRNAs, which then are processed by DCL2 and DCL4 to produce 21-22-nt siRNAs. These siRNAs form a complex with AGO1 to direct Post Transcriptional Gene Silencing (PTGS) or with AGO2, NEEDED FOR RDR2-INDEPENDENT DNA METHYLATION (NERD), Pol V and DRM2 to direct *de novo* methylation (Mari-Ordonez et al., 2013; Matzke and Mosher, 2014; Nuthikattu et al., 2013) (Figure 9).

The involvement of the RdDM pathway in epigenetic response to environmental stresses has been reported by several studies. In *Arabidopsis,* the retrotransposon ONSEN is transcriptionally activated when plant are subjected to heat stress. ONSEN transcription and transposition is affecting the heat responsiveness of nearby genes, providing plants with novel transcriptional regulation in response to heat stress. Heat-induced ONSEN accumulation is enhanced in RdDM mutant, thus suggesting that RdDM repressed ONSEN activity (Ito et al., 2011; Pecinka et al., 2010). In a recent study Yu et al (2013) reported that the bacterial flagellin peptide elicitor flg22 treatment could trigger de-repression of RdDM targets including ONSEN and retroelement *AtSN1*. Interestingly, flg22-induced *AtSN1* activation depends on REPRESSOR OF SILENCING 1 (ROS1) mediated demethylation, suggesting relation between RdDM and ROS1 in epigenetic response to stress (Yu et al., 2013).

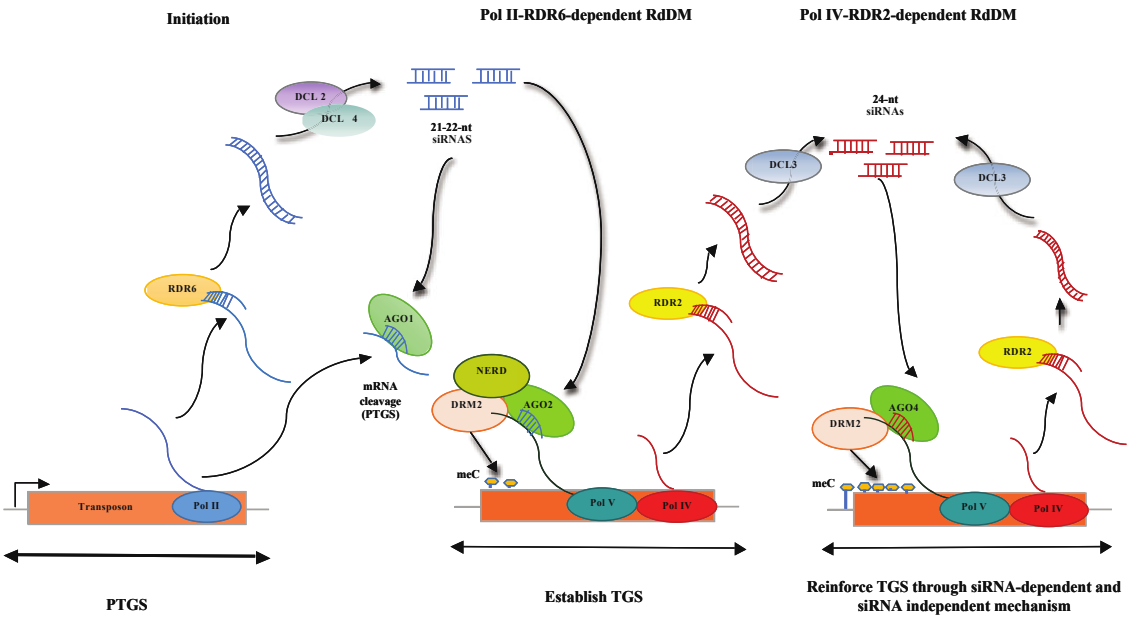


**Figure 9. RNA-directed DNA methylation pathway.** DNA methylation by RdDM pathway could be guided by 21-22-nt or 24-nt siRNAs. In 21-22-nt siRNAs guided DNA methylation, a newly inserted transposon is transcribed by Pol II and the transcript is copied by RDR6 to produce dsRNAs. These dsRNAs are cleaved into 21-22-nt siRNAs by DCL2 and DCL4 and bound to AGO1 to induce PTGS or to AGO2 to induce *de novo* methylation. In 24-nt siRNAs guided RdDM, repetitive regions are transcribed by Pol IV and the transcript is converted to dsRNAs by RDR2. These dsRNAs are processed by DCL3 to produce 24-nt siRNAs, which are then loaded into AGO4. siRNAs-AGO4 complex interacting with Pol V and DRM2 to induce *de novo* methylation.

Plants defective in components of the RdDM pathway are hypersensitive to heat stress that correlates with the miss-regulation of heat-responsive genes. The miss-expression of these genes was attributed to the impaired epigenetic regulation of TEs flanking them (Popova et al., 2013). Similarly, Dowen et al (2012) reported that *Pseudomonas syringae* infection induced hypomethylation of discrete TEs and was associated with transcriptional change of several defence-related genes. Moreover, RdDM mutants infected with *P. syringae* miss-regulated these genes, again indicating that RdDM-induced methylation is associated with stress responses in plants (Dowen et al., 2012). Further, improved tolerance in the progeny of plants subjected to salt stress (Boyko et al., 2010), pathogen infection (Luna and Ton, 2012), methyl jasmonate application and herbivory attack (Rasmann et al., 2012) that are associated with DNA methylation changes are impaired in RdDM mutants, thus suggesting that RdDM is required for both maintenance and inheritance of epigenetic changes induced by stress. In summary, there is considerable evidence supporting the view that both siRNAs and the RdDM pathway are involved in the regulation of DNA methylation directed by environmental stresses.

### 4.1.3 Heritability of Stress-induced DNA Methylation Changes

Some of stress-induced DNA methylation changes could be retained and maintained across mitotic divisions, allowing plants to respond better to subsequent stress exposures. Therefore, through stable epigenetic modifications plants can become "primed" and develop enhanced tolerance to stress. Heritable epigenetic changes associated with stress have been reported in plants (Kinoshita and Seki, 2014; Pieterse, 2012). However, a major barrier for the inheritance of stress-induced

epigenetic changes is the reprogramming taking place during plants gametogenesis and embryogenesis. During this reprogramming the majority of DNA methylation changes acquired during plants lifetime are reset (discussed at section 1.5 and section 1.6) (Gutierrez-Marcos and Dickinson, 2012; Kawashima and Berger, 2014). The degree to which epigenetic reprogramming may limit stress-induced transgenerational epigenetic inheritance is still unclear. However, results from several studies have suggested that epigenome reprogramming in plants is a "leaky" process. Several studies have reported that epigenetic changes acquired by stressed parents can be passed down to the non-stressed progeny. For example, Bilichak et al. (2012) reported that compared to progeny of control plants the progeny of salt stressed plants displayed genome wide hypermethylation (Bilichak et al., 2012). In another study, following 10 generations of salt stress treatments Jiang et al. (2014) showed that the majority of salt induced methylated changes accumulated in parental plants were inherited across two non-stressed progenies (Jiang et al., 2014). In the case of biotic stress, infection with pathogen *P. syringae* increased pathogen resistance in the progeny for three successive generations. This acquired resistance was accompanied by inherited hypomethylation in the progeny, especially in sequences flanking defense-related genes (Luna et al., 2012). In summary, environmental stress triggers methylation changes at particular loci in the genome that could form stable epiamutations. These epimutations could be maintained through mitosis and meiosis and inherited over multiple generations escaping from the epigenetic reprogramming taking place during sexual reproduction.

### 4.1.4 Chapter Aims

The aim of this chapter is to characterize DNA methylation changes induced by multigenerational salt stress treatments and to assess their stability across generations. In addition, I will assess the relationship between DNA methylation and other epigenetic modifications in response to salt stress.

## 4.2 Results

### 4.2.1 DNA Methylation Changes in Response to Salt Stress

In chapter 3, I found that progenies of plants with repeated exposure to salt stress shows a transient increase of tolerance to salt stress. The inheritance of this acquired trait was impaired in mutants defect in DNA methylation and demethylation pathway (Figure 5 and 8). To assess whether the increased tolerance observed was linked to DNA methylation changes, I performed a whole-genome bisulfite sequencing analysis of control, 25 mM NaCl and 75 mM NaCl treated plants in generations 1, 3 and 5. To evaluate the stability and inheritance of the salt-induced methylation changes I also performed bisulfite sequencing on P1 and P2 plants derived from either control or salt-treated parents. To ensure statistically robust results and excluded inter-individual epigenetic variation that can arise over the course of several generations (Becker et al., 2011; Schmitz et al., 2011), for each treatment group I collect duplicate samples (two technical replicates) of rosette leaf tissue pooled from 10 plants.

The first analysis conducted was the identification of differentially methylated positions (DMPs, an individual cytosine position displaying significant methylation rate changes) by performing pairwise comparisons between two samples (Figure 10). Because plants from generation 1, 3 and 5 had been grown at different times point, to eliminate false DMPs coming from spontaneous methylation changes across generation and fluctuating growth conditions, I only compared samples belonging to the same generation. I found that salt-induced DMPs are rare, with on average only 6,866 DMPs detected when comparing control and salt treated plants in each generation. Despite the rare occurrence of salt-induced DMPs, in all generations,

Principal component analysis (PCA) and hierarchical clustering based on methylation rates still grouped salt-treated sample (stressed-P0 samples) together, separated from control, P1 and P2 samples (Figure 10). These data indicate that multigenerational salt stress treatment has minor effects on the genome-wide DNA methylation status of plants at individual cytosine positions, however it still inducing enough changes to separate DNA methylation pattern between salt-treated and control plants. The P1 and P2 samples are grouped closer to non-treated control than to the treated parental plants (Figure 10), suggesting that methylation changes at individual cytosine level are erased when the stress is elevated and not passed to the next non-stressed generations, even after salt treatment for five constitutive generations.

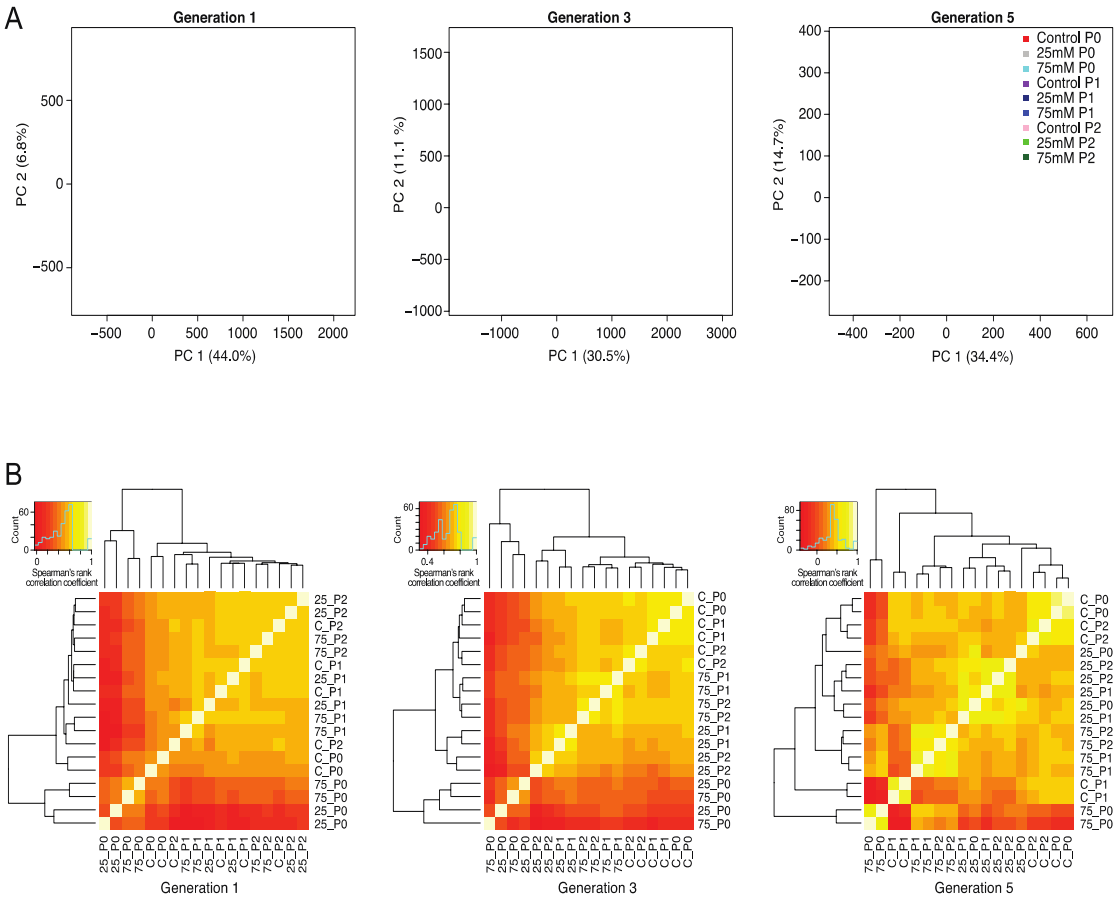


**Figure 10. Analysis of salt-induced differentially methylated positions (DMPs)** (A) Principal component (PC) analysis of methylation rates at differentially methylated positions (DMPs) identified in each generation. Numbers in brackets indicate the percentage of variation explained by the respective PC. (B) Bi-hierarchical clustering of pairwise correlation analyses based on DMP methylation rates.

In this experiment I found considerably less DMPs than previously described by Jiang et al. (2014). They identified 24,655 DMPs when comparing individual control plant with plant that had been treated with salt over ten generations, whereas following five generations of salt stress treatment on average I only detected 6,866 DMPs (Jiang et al., 2014). My result also indicates that most of salt-induced DMPs are erased in the non-stressed progeny. In contrary, Jiang et al. (2014) reported that majority of salt induced CG-DMPs (~75%) were inherited across two non-stressed progeny (Jiang et al., 2014). To compare the two datasets I re-analysed the sequencing data published by Jiang et al. (2014). Hierarchical clustering analysis and PCA of the published data indicated that between individual replicates only few DMPs reported were consistently induced by salt stress. The salt-treated samples (G10_S) were not grouped together and clustered closer to control samples (G10_C) rather than to each other (Figure 11A). This results suggest that the majority of DMPs identified in this study orginated from individual variation and appeared to be of stochastic origin. Therefore, most of the DMPs described could not be attributed as specific response to salt stress.

DNA methylation changes could occur at individual cytosine positions or at contiguous stretches of sequence. A recent study has shown that differentially methylated positions (DMPs) behave distinctly from differentially methylated regions (DMRs) (Hagmaan et al., 2015). DMPs are commonly found at sparsely distributed CG sites within gene bodies while DMRs mostly occur at densely methylated areas such as centromeric and pericentromeric regions. In addition DMRs could be found at various genomic contexts (both genic and intergenic) and more often found overlapped with TEs rather than genes (Hagmaan et al., 2015).

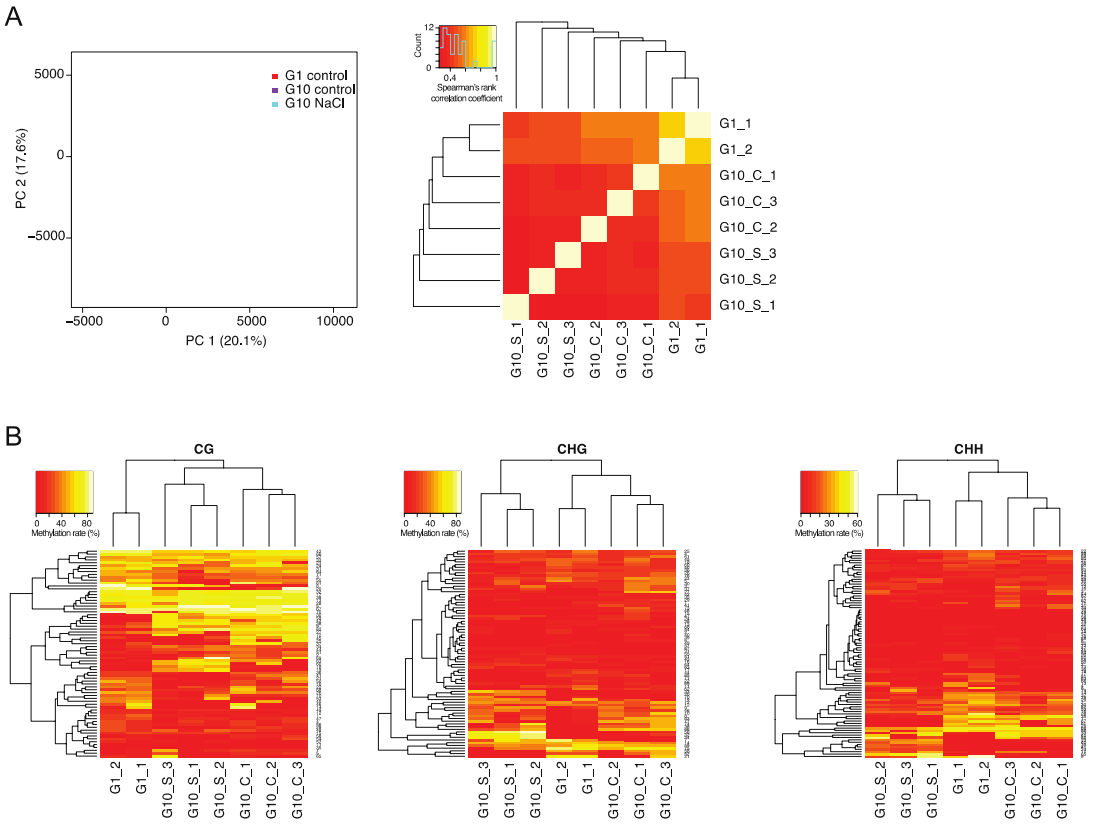


**Figure 11. DNA methylation variation after ten generations of salt-treatment. All analyses are based on data from Jiang et al. (2014)** (A) Principal component (PC) analysis of methylation rates at differentially methylated positions (DMPs). Numbers in brackets indicate the percentage of variation explained by the respective PC. Bi-hierarchical clustering of pairwise correlation analyses based on methylation rates at differentially methylated positions (DMPs) identified in all pairwise comparisons and with full information across all samples (right panel). (B) Bi-hierarchical clustering based on DMR methylation rates, divided by sequence context. The methylation rate of each DMR per sample was calculated as the average methylation rate of cytosines contained in that region. Only DMRs covered in each sample were considered. (G1: generation 1; G10: generation 10; C: control; S: salt-treated)

I identified on average 24,700 methylated regions (MRs) per sample with a median length of 272 bp (mean: 856 bp). To identify DMRs between samples within generation, I grouped samples of the same generation and of the same treatment together and considered them as replicates. P0 control, P1 control and P2 control samples were grouped as "non-stressed"; P0 salt-treated samples as "stressed-P0"; P1 samples derived from salt-treated P0 plants as "stressed-P1"; and P2 samples derived from salt-treated P1 plants as "stressed-P2". Tests for DMRs were then performed between these four groups. Previous studies have shown that salt stress could lead to genome-wide methylation changes (Bilichak et al., 2012; Jiang et al., 2014), however my analysis revealed that the salt-induced DMRs are rare. Following five generations of constitutive salt-stress treatment I only identified 49 salt-induced DMRs in generation 1, 148 DMRs in generation 3 and 153 DMRs in generation 5 (full list of DMRs can be found in Appendix Table 6). Among the identified DMRs several were recurred in generation 3 and 5 (24 DMRs) and only a few recurred between generation 1 and 3 (7 DMRs) or generation 1 and 5 (4 DMRs). Intriguingly, there is no DMR found to be overlap among all generations.

For all three generations tested salt-induced DMRs are mainly annotated to TEs and intergenic regions. Interestingly, compared to random methylated regions, I found that the salt-induced DMRs are over-represented by three- to seven-fold in 2-kb-regions upstream or downstream of gene transcription start sites, suggesting that salt-induced DMRs are rare but targeted to specific genomic regions (Figure 12A).

An unsupervised DMR-based hierarchical clustering analysis grouped together salt stressed-PO samples together in all generations, similar to results I obtained from DMP-based PCA and clustering analysis. In generation 1, stressed-P1 and stressed-P2 samples are grouped together with non-stressed control samples. However, in

generations 3 and 5 the stressed-P1 samples formed another sub-group, separated from remaining stressed-P2 and non-stressed control samples (Figure 12B). These results were in accordance with the phenotyping data described at previous chapter (section 3.2.2), in which the salinity tolerance differences between progenies of control and salt-treated plants became apparent only after the parents had experienced salt stress treatment for two or more generations, and were not detectable if the parental plants had been subjected to salt stress for only one generation. My DMRs analysis also showed that in all generations, P2 plants showed a methylation pattern similar to that of the control plants, suggesting that most of salt-induced DMRs are erased in the subsequent non-stresses generations. These results again concur with my phenotypic data, where the increased tolerance to salinity displayed by P1 plants was lost in the P2. Taken together my analysis revealed that multigenerational salt stress treatment induces transient DNA methylation changes at specific genomic regions and that these epigenetic changes correlate with transient adaptive phenotypic changes in plants.

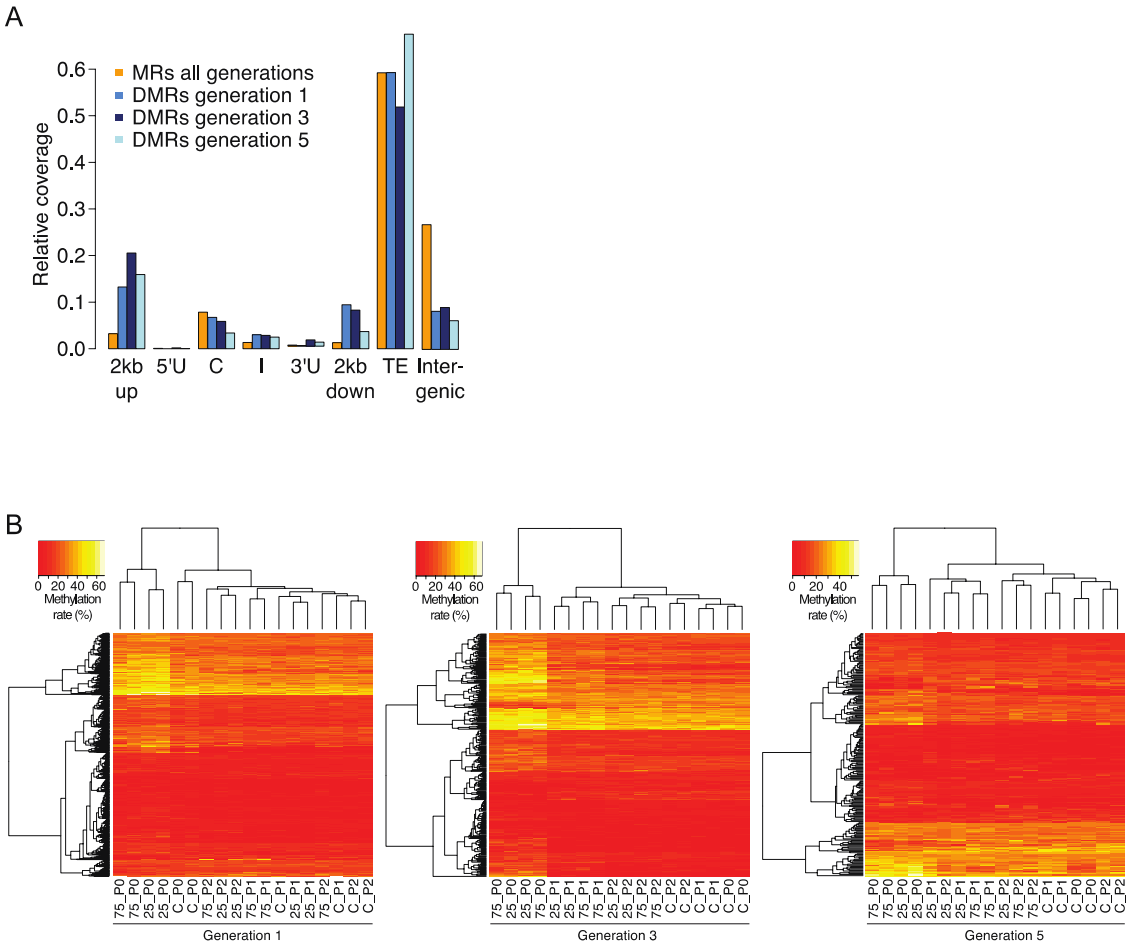


**Figure 12. Analysis of salt-induced differentially methylated regions (DMRs)** (A) Annotation of cytosines in MRs and DMRs between P0 control and salt-treated samples. All positions in the respective class of regions were assigned to one annotation in the following order: CDS (C) > intron (I) > 5'UTR (5'U) > 3'UTR (3'U) > 2 kb upstream > 2 kb downstream > transposon (TE) > intergenic. All MRs identified in the different samples were collapsed into a unified set before annotation. (B) Bi-hierarchical clustering based on DMR methylation rates, divided by generation. The methylation rate of each DMR per sample was calculated as the average methylation rate of cytosines contained in that region. Only DMRs covered in each sample were considered.

There was no significant difference in the average CG methylation rate of DMRs between control and salt-treated samples ($P > 0.05$ in all generations, unpaired Student's *t*-test). However, methylation rate in the CHG and CHH contexts inside DMRs was significantly higher in salt-treated P0 samples compared to control samples ($P << 0.01$ in all generations, unpaired Student's *t*-test) (figure 13). This result indicate that salt stress lead to hyper-methylation of genomic regions at CHG and CHH sites. To validate these results I analysed published DNA methylation data from individual plants exposed to salt for 10 generations (Jiang et al., 2014). When focusing the analysis on DMRs and when three individual samples reported in the study were treated as replicates, similar to my result, I found that only methylation changes in CHG and CHH context are correlated with salt treatment, whereas changes in CG methylation were stochastically distributed among samples (Figure 11B). In generation 1 and 3, methylation rate differences between control and salt treated plants were only observed in P0 plants but not in P1 and P2 plants, indicating that majority of methylation changes in the DMRs were being reset to basal level in the non-stressed progeny. However, in generation 5 methylation rate differences between control and salt treated plants were still observed in the P1, which then erased in the P2 (Figure 12). These results suggest that continuous multigenerational exposure to salt stress may lead to stable methylation changes in the genomic regions, that can not immediately be erased by epigenetic reprogramming during sexual reproduction, thus allowing its transmission to the direct non-stressed progeny.

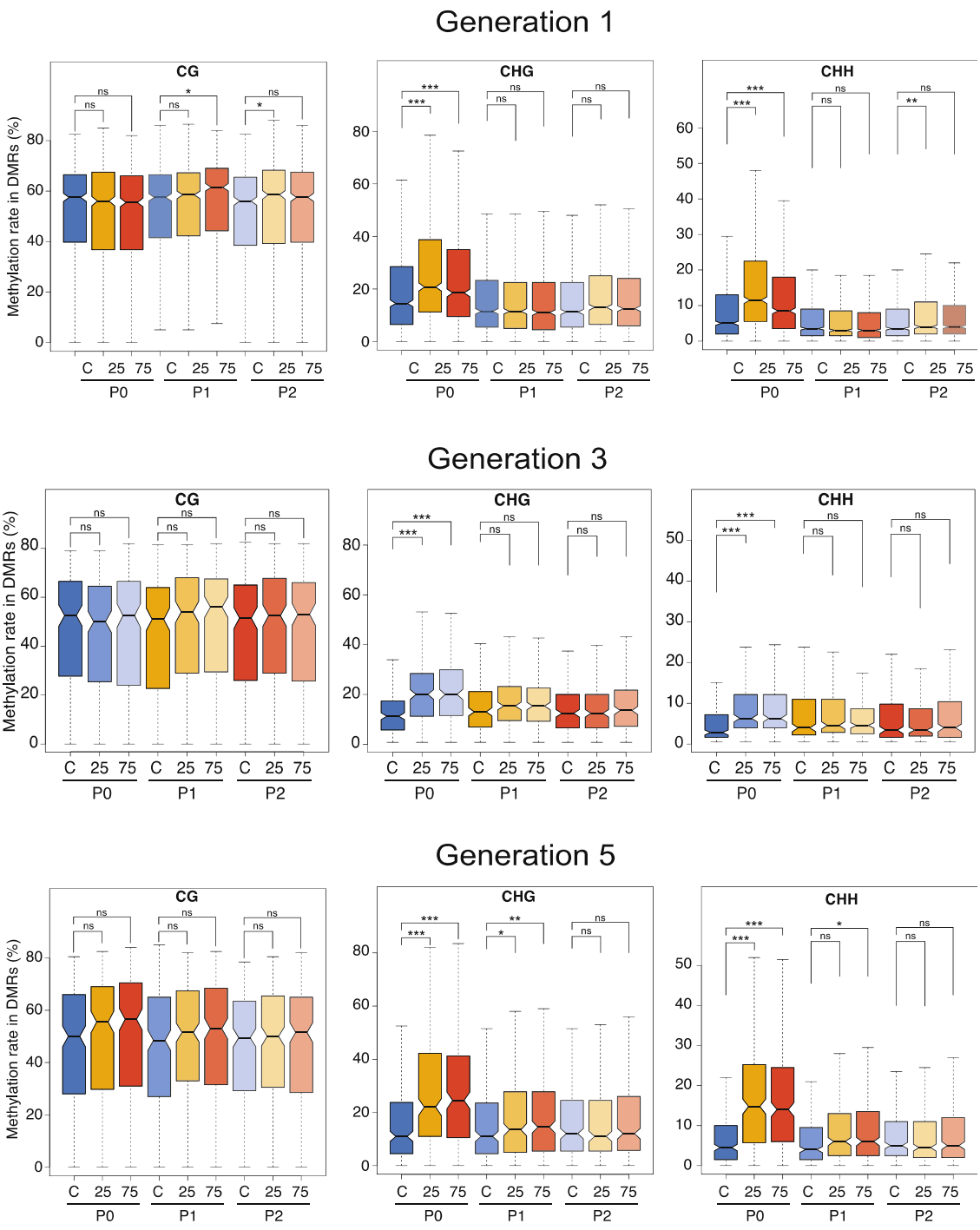


**Figure 13. Methylation rates of salt-induced DMRs in generation 1, 3 and 5.** Methylation rates by sequence context in DMRs identified between control and stress-treated plants (P0), and the derived P1 and P2 plants from each generation. "25"=25 mM NaCl; "75"=75 mM NaCl; "C"=Control (*** p<0.001; ns p>0.05; unpaired two-tailed Student's t-test).

### 4.2.2 Salt Stress Induces Specific Hypo- and Hypermethylation Changes

Several studies have reported that salt stress caused gain rather than loss of methylation in the genome (Bilichak et al., 2012; Boyko et al., 2010; Jiang et al., 2014). Similar to previous report, I found that most of salt-induced DMRs identified between stressed-P0 and non-stressed control samples (~81%) are hypermethylated. This effect was strongly observed in generation 3 and 5 where salt stress induced higher rates of methylation in the DMRs (Figure 14). Interestingly, in generation 1 only a small amount of methylation changes were observed between non-stressed control and stressed-PO plants. In addition, in generation 1 hypo- and hyper-methylated DMRs occurred equally (Figure 14A and B). These data suggests that the first generation of stressed plants is epigenetically less responsive and explains the lack of adaptation to salt stress (Figure 5 and 6). It also suggests that multi-generational salt stress lead to elevated response to stress and accumulation of hypermethylated DMRs in the *Arabidopsis* genome. In all generations tested, salt stress induces hypermethylation at specific contexts. Salt-induced CG hypermethylation showed stochastic variation across P0, P1 and P2 plants, while CHG and CHH hypermethylation were significantly higher in stressed-P0 plants then gradually reverted back to control level in P1 and P2 plants (Figure 14A and B). The CHG and CHH-specific hyper-methylation in the stressed-P0 plants suggest the involvement of the RdDM machinery in salt stress response, in agreement with the lack of salt tolerance increase found in the P1 of *cmt3* and *nrpd1a* mutants (Figure 8).

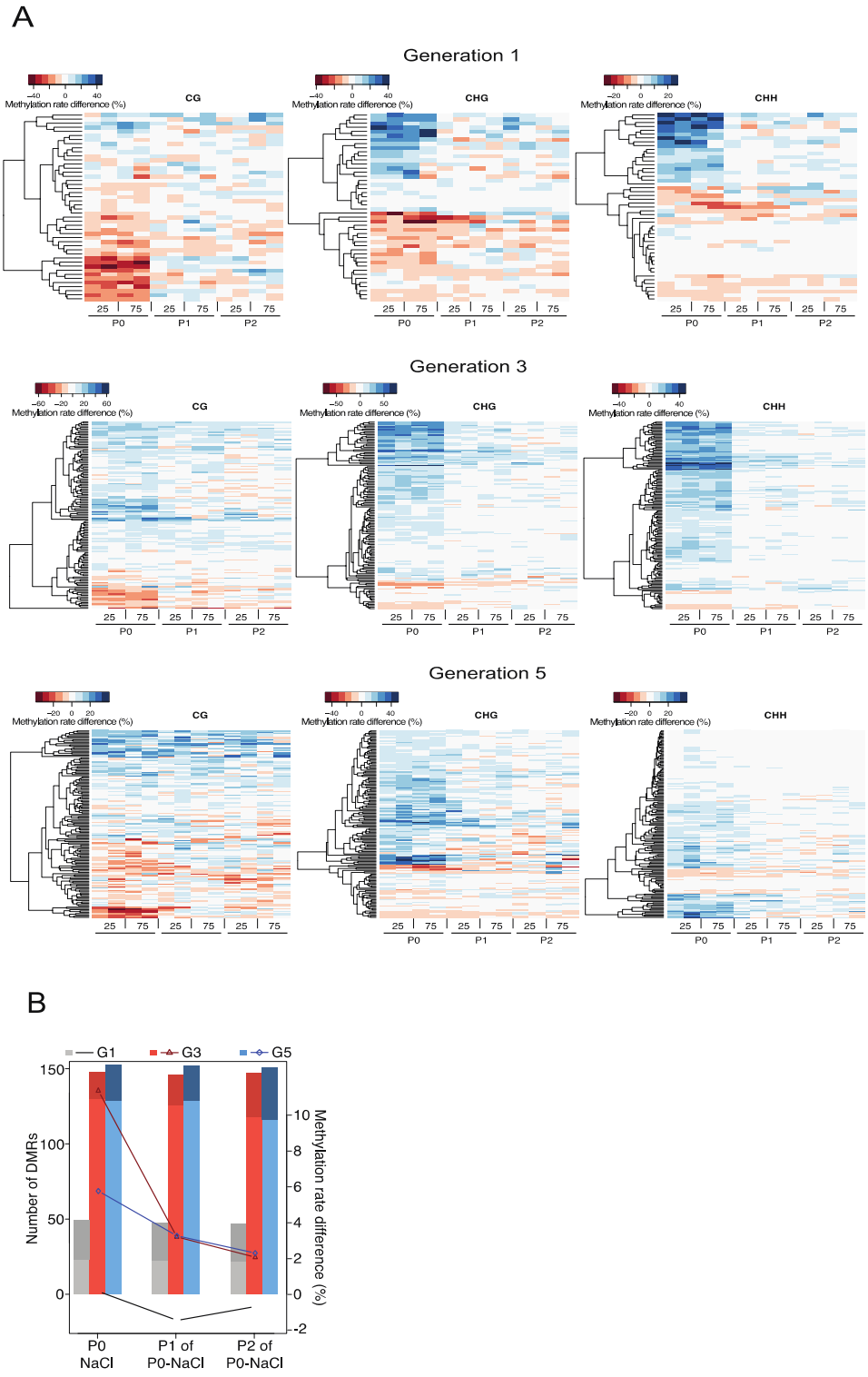


**Figure 14. Dynamics of methylation rate changes in DMRs.** (A) One-directional clustering of DMRs in generation 1, 3 and 5 by methylation rate difference, divided by sequence context. Blue color indicates hyper-, red color indicates hypo-methylation (B) Barplots show the number of DMRs that are either hypo- (dark colour) or hyper-methylated (light colour) in the salt-treated P0 and the subsequent P1 and P2 generations compared to the combined control samples. Line plots indicate the net methylation rate change in DMRs. (**G1: Generation 1; G2: Generation 2; G3: Generation 3**)

Hypo- and hypermethylated DMRs mapped to different genomic regions. Hypermethylated DMRs were more often overlapping with or in proximity to TEs, whereas hypomethylated DMRs occurred more frequently overlapping with or close to genes (Figure 15A). Transposable elements are frequently found overlapping with 24nt-siRNA generating loci. These small RNAs are generated from TEs and tandem repeats whose major role is to repress transposon activity by methylation to maintain genome integrity (Bond and Baulcombe, 2015). Although salt-induced hypermethylated DMRs were found in closer proximity to TEs than hypomethylated DMRs, there are no different in distance to 24nt-siRNA loci between them (Figure 15B). The hypo- and hyper- methylated DMRs also overlapped with different type of TE family. The hypermethylated DMRs were found in proximity to Helitrons, a TE family that known as a target site for RdDM machinery (Nuthikattu et al., 2013). While hypomethylated DMRs occurred close to Copia, HAT, and Line_L1 TEs family (Figure 15C). Previous studies have reported in *Arabidopsis* that these three TEs families and DNA demethylases are implicated in the positive regulation of stress responsive genes (Le et al., 2014). Collectively, these results suggest that salt stress induces transient hypo- or hypermethylation changes at non-CG sites in transposons flanking coding genes.

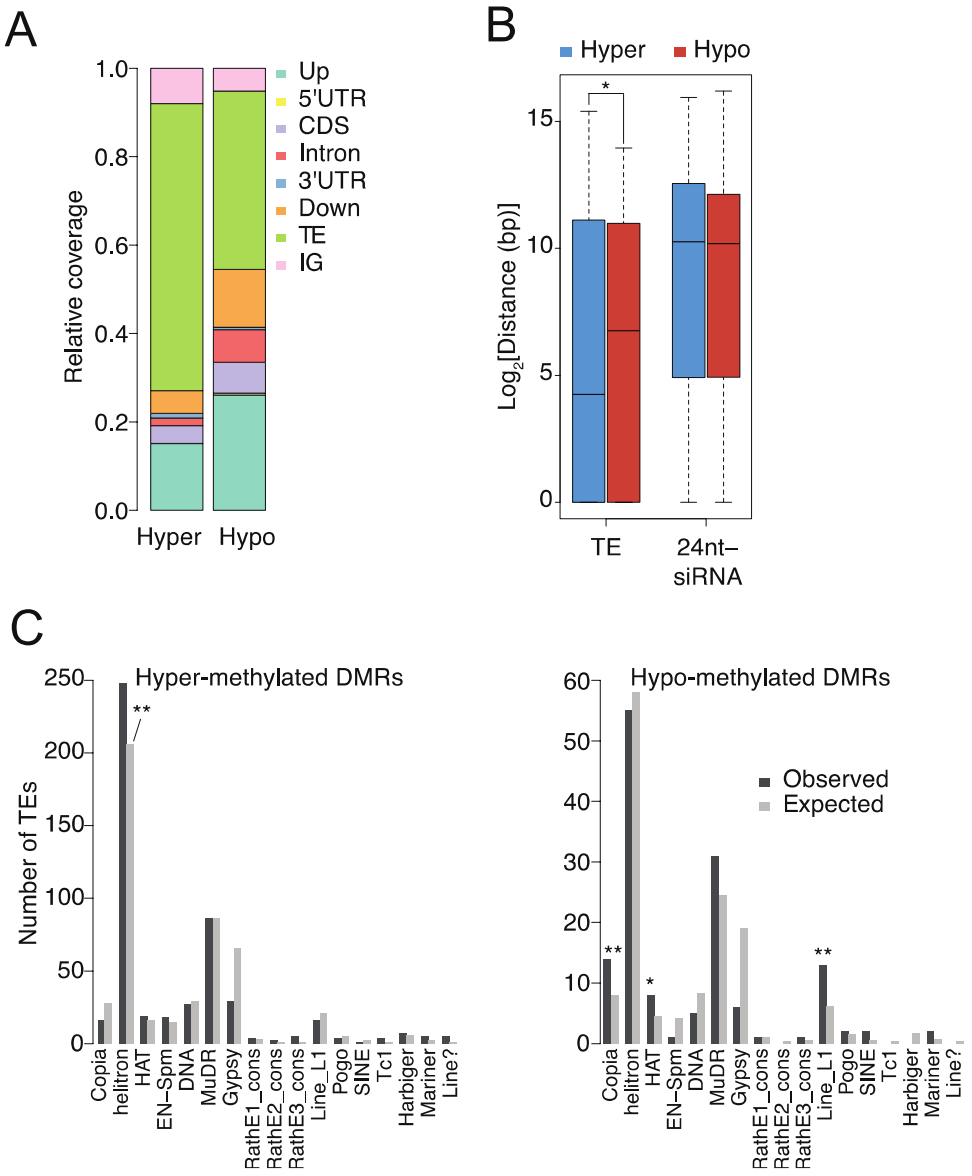


**Figure 15. Hypo- and hyper- methylated DMRs are annotated to different genomic region.** (A) Annotation of hyper- and hypo-methylated DMRs (Up: 2 kb upstream; Down: 2 kb downstream; IG: intergenic). (B) Distance of hyper- and hypo-methylated DMRs to the most proximal transposable element (TE) or 24 nt-siRNA locus (original data from Fahlgren et al., 2010). Horizontal bar corresponds to median, whiskers indicate entire 75$^{th}$ percentile; outliers are not shown (* $p<0.05$, unpaired Student's *t*-test). (C) Classes of TEs in proximity to hyper- and hypo-methylated DMRs. ("25"=25 mM NaCl; "75"=75 mM NaCl; "C"=Control; "Gen"=generation)

### 4.2.3 Salt Stress Induces Methylation Changes at Labile Regions of Plants Genome

Following multigenerational salt stress treatment, I was able to identify a substantial number of salt-induced DMRs, however only few of them were recurrently found across three different generations tested. This observation suggests that methylation changes in response to salt stress are not consistently targeted to specific genomic regions. To determine the significance of salt induced DMRs identified in this study, I compared my salt-induced DMRs data with published DMRs data following cold-stress treatment in *A. thaliana* seedlings (Seymour et al., 2014), however I failed to detect an overlap between these two datasets. Nonetheless, I found a 49% overlap between DMRs induced by salt stress with DMRs that arise spontaneously in mutation accumulation (MA) lines grown under controlled environmental conditions (MA-DMRs) (Becker et al., 2011) (Figure 16A). Such spontaneous DMRs are often found in more than one individual, pointing to specific regions of the genome being particularly susceptible to epigenetic changes. These results indicate that there are regions in *Arabidopsis* genome that are prone to methylation changes and that salt stress exposure could quickly destabilise the epigenetic state of this labile regions of the genome. Further, I evaluated whether DMRs overlapping with MA-DMRs had different characteristics from those that not overlapping with MA-DMRs (Figure 16B). Both groups behaved similarly in a clustering analysis in generations 3 and 5 (but not in generation 1), with salt-stressed-P0 samples clustering in one group and control and untreated progenies in another, indicating that they were not fundamentally different and both of them are correlated with the salt stress treatment. Collectively these results suggest that salt-stress exposure lead to methylation changes in epigenetically labile regions of the plant genome.

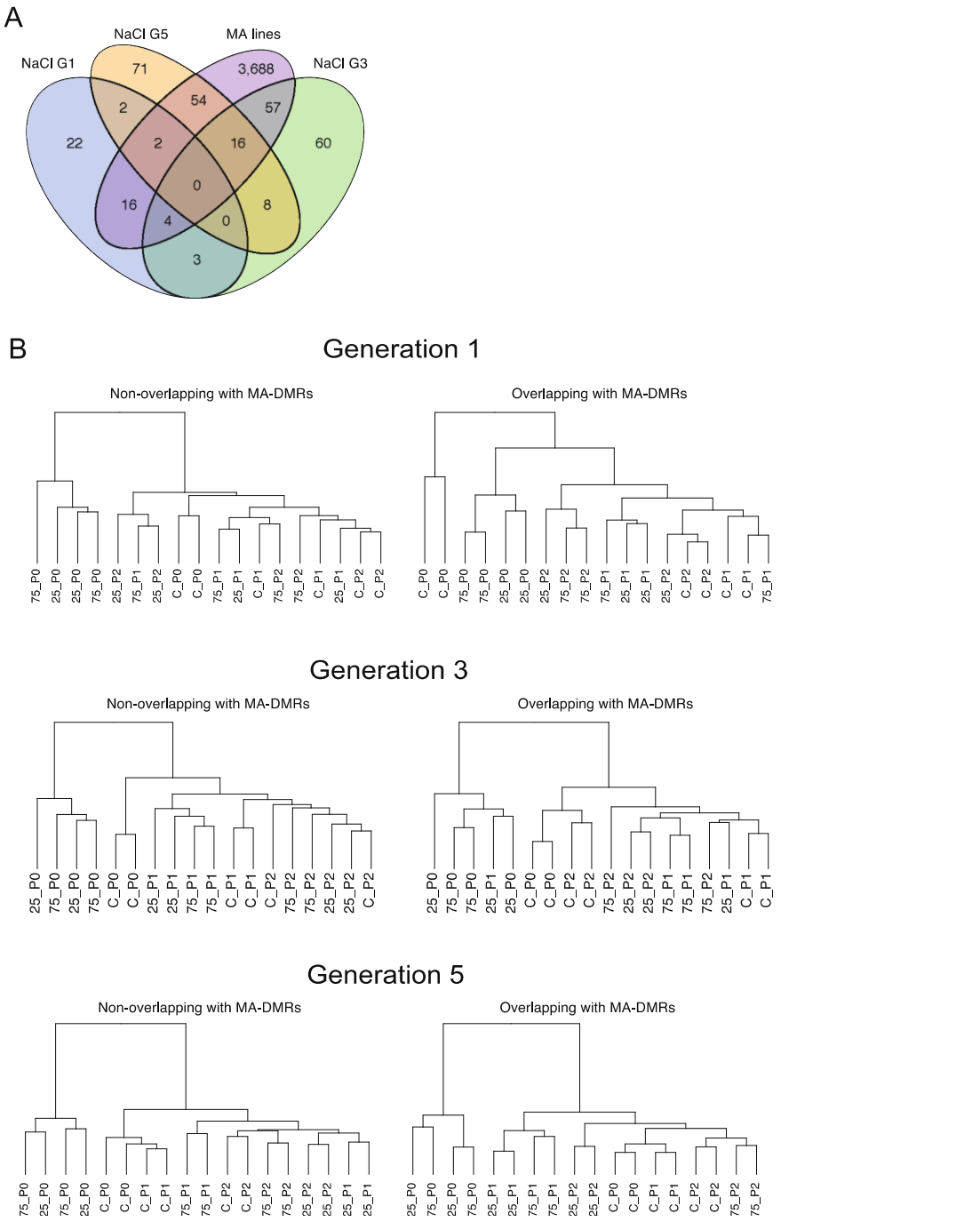


**Figure 16. Overlap between salt induced DMRs and MA-DMRs** (A) Venn diagram showing the overlap (including 500 bp flanking windows) among DMRs between P0 control and salt-treated samples from each generation and with DMRs from a previous analysis of mutation accumulation (MA) lines (B) Clustering analysis of DMRs between control and salt-treated samples in generation 1,3 and 5 that overlap or do not overlap with MA-DMRs. ("25"=25 mM NaCl; "75"=75 mM NaCl; "C"=Control).

### 4.2.4 Salt-induced DNA Methylation Changes Correlate with Histone Methylation Marks

Beside DNA methylation, salt stress responses also involve changes in chromatin. Sani et.al (2013) has shown that in *Arabidopsis*, exposure to salt stress in early stages of plant development leads to shortening and fractionation of H3K27me3 islands, and that these changes correlated with transcriptional changes of salt-responsive genes (Sani et al., 2013). To assess if a relationship between DNA methylation and chromatin marks exist, I compared the identified salt-induced DMRs with chromatin changes associated with salt stress treatment. I found 35% overlap between salt-induced DMRs and salt-induced changes in H3K27me3 occupancy. This correlation was especially strong between hyper-methylated DMRs and decrease in H3K27me3 occupancy (38%). Collectively, these results suggest that DNA methylation and chromatin modifications directed by salt stress are both targeted to the same labile regions of the genome.

**Table 2**. Intersections between High Salinity-induced differentially methylated regions (HS-DMRs), Methylated regions (MRs) and chromatin marks. The number underneath chromatin mark indicates the number of regions with a significantly increased (+) or decreased (-) representation after salt stress. DMR are split into hyper or hypo methylated and the number of intersections is indicated with bold numbers and percentage.

| **Chromatin mark** | **HS-DMRs** | | | | **MRs** | |
|---|---|---|---|---|---|---|
| | Hypo_meC | | Hyper_meC | | | |
| Total # regions | 70 | | 280 | | 72,074 | |
| | + | - | + | - | + | - |
| H3K4me2 | **0** | | **3** | | **663** | |
| (+650)(-98) | 0 | 0 | 3 | 0 | 508 | 155 |
| H3K4me3 | **4** | | **8** | | **412** | |
| (+1454)(-46) | 4 | 0 | 8 | 0 | 1,065 | 40 |
| H3K9me3 | **0** | | **0** | | **1,051** | |
| (+276)(-254) | 0 | 0 | 0 | 0 | 484 | 920 |
| H3K27me3 | **18 (25%)** | | **106 (38%)** | | **9,092 (13%)** | |
| (+1213)(-6520) | 3 | 15 | 7 | 99 | 1,318 | 7,774 |

### 4.2.5 Inheritance of Salt-induced DNA Methylation Marks is Under Parent-of-Origin Control and Regulated by DEMETER.

Although the majority of salt-induced methylation changes in stressed-P0 plants were reverted back to basal level in the untreated P1 progeny, a small amount of these changes, especially in generation 3 and 5, are still retained in P1 plants. These inherited methylation marks might be responsible for the increased tolerance observed in the P1 progeny of salt-treated plants. Several studies have suggested that the inheritance of stress memory in plants may be under maternal control (Agrawal, 2001; Pecinka and Mittelsten Scheid, 2012). To investigate further whether the increased tolerance in P1 progeny is under parent of origin control, I performed reciprocal crosses between salt-stressed plants with non-stressed control plants. To test their tolerance level, seeds obtained from these reciprocal crossing were germinated and grown in media with high salinity. Only when the mother plant had been exposed to the salt stress did the P1 become more tolerant to high salinity, indicating that transgenerational response to salt stress were inherited maternally, but not paternally transmitted (Figure 17).

Phenotypic data suggest that salt induced methylation changes might be erased in male gametes. Erasure and reprogramming of methylation marks in male gametes occurs through the action DNA glyclosylase DEMETER (DME). To investigate whether inheritance of salt-induced methylation marks and increased tolerance is mediated by DME, I subjected DME mutant lines (*dme-6*) to salt stress for two successive generations and tested their progenies for tolerance of high-salinity. I found that the progeny of stressed *dme-6* plants showed better tolerance to high salinity compared to the progeny of stressed Col-0 plants or control plants (Figure 17). These data indicate that the resetting of salt-induced DNA methylation marks in

male gametes is primarily mediated by DME, thus ensuring the removal of methylation marks to the offspring.

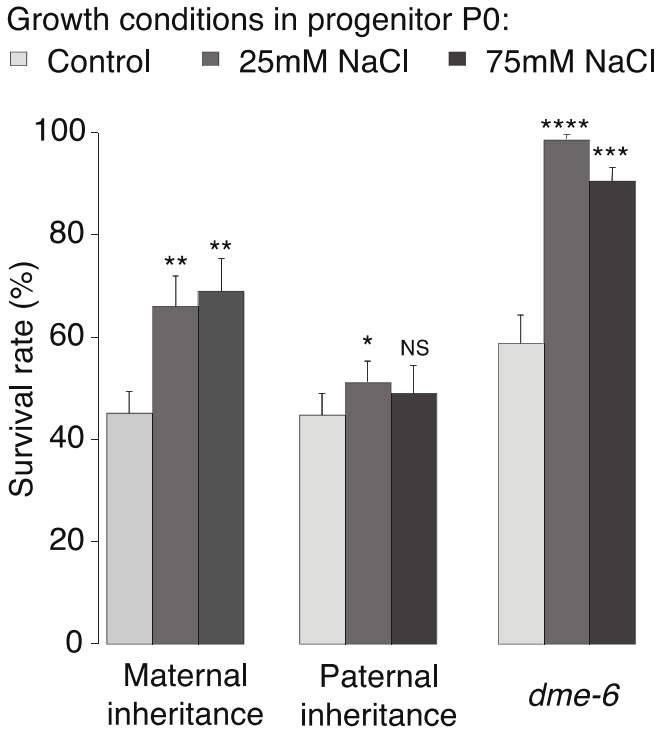


**Figure 17. Maternal and paternal inheritance of acquired tolerance following salt stress**. Survival rate on 150 mM NaCl of seedlings derived from crosses in which either the paternal or the maternal plant had been exposed to salt stress (left panel). Survival rate of progeny of *dme-6* plants on medium with 150 mM NaCl. Survival rates are shown in percentage (average of three plates, calculated from 50 seeds per plate). Asterisks indicate significant difference to the respective control (unpaired Student's *t*-test; ** $p<0.01$; * $p<0.05$; NS $p>0.05$) .

To investigate whether DNA methylation changes in somatic tissue are erased in the male gametes following gametogenesis, I performed whole genome bisulfite sequencing on isolated sperm cells (SC) and vegetative nuclei (VN) collected from control and salt-treated plants. In accordance with my phenotypic analyses, neither SC nor VN asccumulated DNA methylation changes upon salt stress treatment (Figure 18A). Only three DMRs were identified when comparing VN of control and salt stressed plants, while comparison between and control and salt stressed SC only result in eleven DMRs, all with low methylation rate differences and limited overlap with somatic salt-induced DMRs (full list of DMRs can be found in Appendix Table 6). However, I found that global DNA methylation patterns different significantly between SC and VN regardless the stress treatment. SC displayed broad distribution of CG and CHG methylation across centromeric and pericentromeric regions, while methylation in VN in all sequence contexts was only observed at centromeric region, suggesting genome-wide hypomethylation in VN (Figure 18D). Comparison between SC and VN results in 13,776 DMRs (SV-DMRs), which predominantly annotated to TEs and were particularly enriched at up- and down-stream regions of genes (Figure 18B and 18C). Interestingly, around 76% of salt induced DMRs in the leaf samples are overlapped with SV-DMRs, suggesting that differential methylation established between SC and VN through the activity of DME might be responsible for erasure of stress memory in the SC (Figure 18B). Collectively my data suggest that transgenerational responses to salt stress are mostly maternally inherited and male gamates undergo extensive erasure of salt-induced methylation marks through DME activity in vegetative cell.

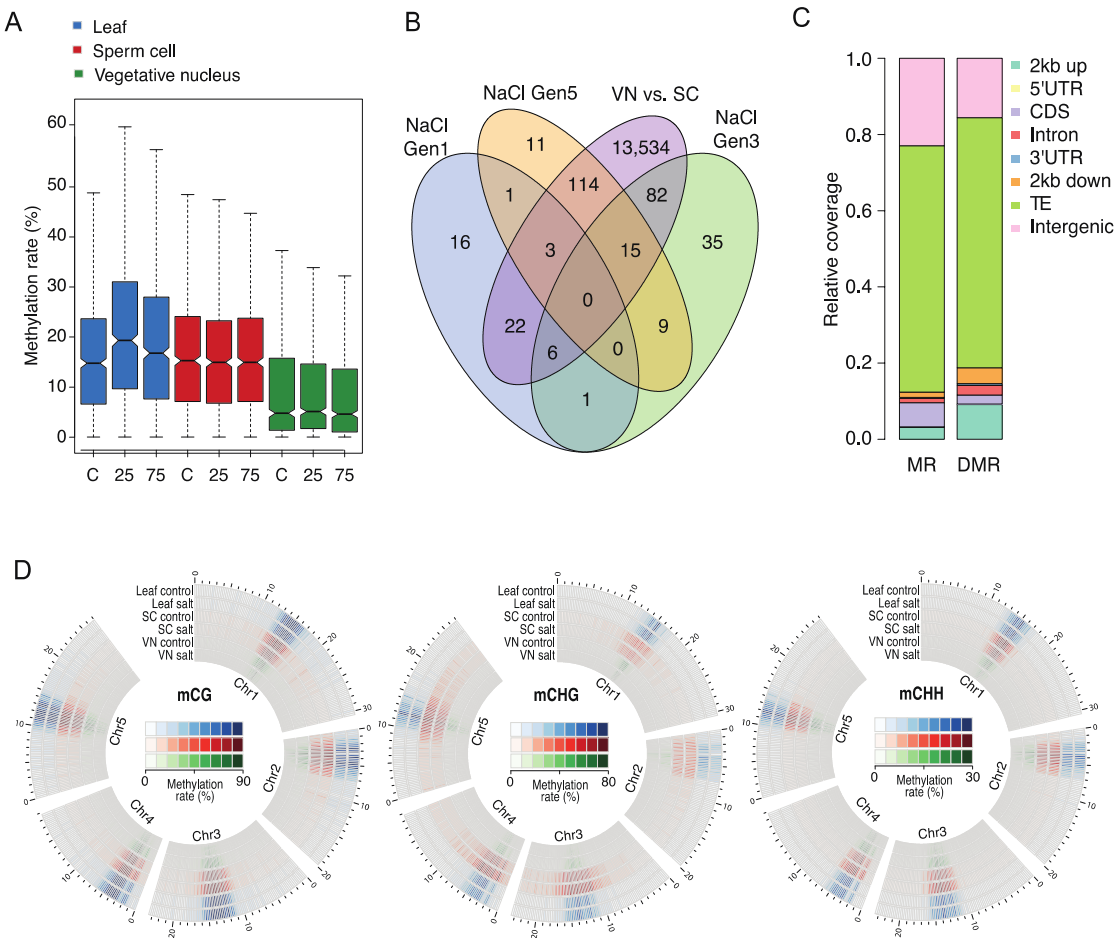


**Figure 18. Parent-of-origin control of stress-induced epimutations** (A) Methylation rates in DMRs identified between control and salt-treated samples in leaves, sperm cells and vegetative nuclei in generation 1 ("25"=25mM NaCl; "75"=75mM NaCl; "C"=Control; "Chr": chromosome). (B) Genome-wide methylation levels in leaf and pollen from control and salt-treated plants in generation 1, divided by sequence context. Methylation rate was calculated as the average methylation rate of cytosines in a 250 kb window. (C) Annotation of the MRs identified in VN and SC, and of DMRs between the two cell types. (D) Overlap of DMRs of the VN-versus-SC comparison with DMRs identified after salt treatment in the three different generations.

### 4.3 Discussion

To evaluate the effect of multigenerational salt stress treatment on the plant epigenome, I exposed *Arabidopsis* plants to salt stress for five constitutive generations and analysed the methylome of the stressed plants and their progenies using whole-genome bisulfite sequencing. To eliminate methylation changes that could arise from environmental fluctuations during growth conditions, I focused the analysis in samples from the same generation. To enable robust statistical analysis and to exclude spontaneous individual epimutations which could arise in the course of few generations (Becker et al., 2011), for each treatment group I collected leaf samples from 10 plants and pooled them for analysis.

My methylome data revealed that multigenerational salt stress treatment induces small methylation changes in the *Arabidopsis* genome, indicating that the effect of salt stress on plants methylation is not genome-wide as previously thought. Nevertheless, the low frequencies of DNA methylation changes I observed showed strong correlation with salt treatment. Clustering analysis based on methylation rate changes clearly separate salt stressed and control samples. In response to salt stress, I observed considerably less DNA methylation changes than reported by previous studies (Bilichak et al., 2012; Jiang et al., 2014). Re-analysis of published data revealed that majority of methylation changes reported in previous study came from individual variation and could not be attributed to a specific effect of the salt treatment. Around 90% of salt-induced DMPs previously reported were identified only in a single plant and were not shared between individual replicates, thus showing that only a few DMPs are consistently induced by salt stress. By using pooled samples my analysis eliminated the stochastic DNA methylation variation

present in individual plants, which has lead to erroneous interpretations in previous reports.

Some stress-induced epigenetic changes are likely to be maintained across mitotic divisions, and may allow plants to respond better to subsequent stress exposures. Through stable epigenetic modifications plants may become "primed" and develop a better tolerance to particular stress (Pieterse, 2012; Prime et al., 2006). Heritable effects accross generations have also been observed, where the offsping "inherit" stress-induced epigenetic changes acquired by the parental. Many studies have reported that stress-induced DNA methylation changes acquired by plants could be inherited across several generations under non-stresses conditions. For instance, Jiang et al., (2014) reported that majority of salt induced DMPs (~75%) were inherited across two non-stressed progeny. Surprisingly, I found that majority of methylation changes detected in stressed parental plants (stressed-PO plants) are erased in the untreated progenies (stressed-P1 and stressed-P2 progenies), suggesting that salt stress applied in my study only had transient effect on plants epigenome. Nevertheless, after 3 and 5 generations of constitutive salt stress treatment the P1-stressed progenies formed a sub-group in clustering analysis separated from P2-stressed progenies and control plants, suggesting that following multigenerational salt stress treatment a small fraction of stress-induced DNA methylation changes are inherited by non-stressed P1 progenies. These data are in accordance with my phenotype data, which shows increased tolerance in the P1 progeny only, thus indicating that acquired DNA methylation changes alter the response of plants to salt stress. Surprisingly, in the P2 progeny these DNA methylation changes are lost, which is associated with a reversion of plant's tolerance to stress back to control level. My data also suggests that one generation of salt stress is not sufficient to

induce transgenerational epigenetic and phenotypic responses. Thus, it appears that repeated exposure to stress within or across generations may be required to trigger sufficient changes in plants epigenome to induce noticeable phenotypic effects.

Tolerance assays performed on salt-stressed epigenetic mutants showed that transgenerational response to salt stress are impaired in mutants defective on DNA demethylation, CHG methylation and RdDM pathway (Figure 8). In agreement with these results, salt stress induced significant methylation changes at CHG and CHH sites, but not CG sites. Most of methylation changes detected were in form of hypermethylation, although in generation 1 hypomethylation was noticeable. The salt-induced CHG and CHH hypermethylation was prominent in P0-stressed plants but then gradually declined in P1 and P2 progenies. Together, these results suggest that transgenerational response to salt stress involved active DNA methylation and demethylation pathway. In response to stress, RdDM may be involved in establishing DNA methylation as well as directing active DNA demethylation. The DNA glycosylase ROS1 is known to preferentially target RdDM-induced methylation sites for demethylation and ROS1 expression is repressed in RdDM mutants (Le et al., 2014; Yu et al., 2013). Recently, Lei et.al (2015) showed that ROS1 expression level is controlled by a helitron transposon element (TE) in the ROS1 gene promoter. This TE is repressed by RdDM, antagonizing it's negative effect on ROS1 expression. Interestingly, this TE is also targeted and self-regulated by ROS1. Thus the balance of DNA methylation and demethylation in the *Arabidopsis* genome appears to be maintained through the antagonistic action of RdDM and ROS1 (Lei et al., 2015).

My methylome data showed that hypermethylation in response to salt stress is targeted to TEs near transcribed genes. Some studies have suggested that the

epigenetic states of TE could affect the neighbouring gene expression. However, it is still unclear how the epigenetic machinery can distinguish TEs from genes. Unique repetitive sequences present in by TEs might play a part in differentiating TEs from other sequences and allow RdDM to recognize these regions specifically. In addition, TEs produce abnormal RNAs, which could be recruited by RdDM to produce siRNAs and provide a self-reinforcing silencing or activating mechanisms (Bond and Baulcombe, 2015). The dynamic methylation state of TEs, the unique sequence it's owned, the ability to move from one location to another in genome and TEs possible function as regulator of gene expression, make them suitable tools for the regulation of adaptive responses to environmental stresses.

Around 49% if DMRs induced by the salt stress overlapped with DMRs that arised spontaneously in mutation accumulation (MA) lines grown for 30 generations under normal growth condition (MA-DMRs) (Becker et al., 2011). It is worth noting that MA-DMRs were identified in individual plants while my salt-induced DMRs were identified from pools of 10 plants. If the MA-DMRs were identified from combinations of 10 individual replicates I expect to find significantly higher overlap between MA-DMRs and salt-induced DMRs. These results show that epigenetically labile regions of the genome are highly sensitive to epigenetic changes, especially in response to stress stimuli. My data clearly shows that prolonged exposure to salt led to an accelerated destabilization of these unstable regions.

Stress memory in form of methylation changes could be inherited through male or female gametes. In my analyses, I found that transgenerational phenotypic effects of salt stress are mostly maternally inherited. In concordance with the observed phenotype, neither Sperm Cell (SC) nor Vegetative Nuclei (VN) showed significant methylation changes in response to salt stress. Interestingly, around 76% of salt

induced DMRs in the leaf samples overlapped with DMRs identified between SC and VN, suggesting that epigenetic responses to salt stress and establishment of differential methylation between SC and VN are regulated by the same pathway. Methylation in CHH context are reported to be lost in the SC following fertilization (Calarco et al., 2012), I postulate that RdDM-dependent salt stress induced hypermethylation at CHH sites are erased in the SC but maintained in female gametes and passed down into the following generation.

In *Arabidopsis* male gametogenesis companion cells (vegetative cells) undergo DNA methylation changes, reactivating TEs activity and increasing siRNAs production, which it is through to be regulated by DME (Gehring et al., 2006; Ibarra et al., 2012; Schoft et al., 2011). Since the genomic and epigenomic state of the companion cells is not directly inherited or affecting the next offspring, it may advantageous for plants to destabilize the epigenomic integrity of these cells to "fine tune" the epigenetic state of reproductive cells (sperm cells). Intriguingly, experimental data indicates that siRNA produced in companion cells might travel to reproductive cells (sperms and egg cells) to establish epigenetic states that are inherited in the progeny (Olmedo-Monfil et al., 2010; Tucker et al., 2012). I hypothesise that the erasure of salt-induced methylation changes in sperm cells could be carried out by DME-dependent siRNAs produced in vegetative cell that travel to sperm cell to direct epigenetic modification of TEs in sperm cells.

## 4.4 Summary

In response to multigenerational salt stress plants accumulate epigenetic changes, non-CG methylation and demethylation, that are targeted to labile regions of genome. These epigenetic changes are associated with a transient tolerance to stress in subsequent generations. Stress tolerance is under parent-of-origin control, likely due to the active epigenetic reprogramming that takes place in the male germline. In the absence of stress, these changes are gradually reset to basal level, suggesting that plants have developed mechanisms to cope with fluctuating environmental conditions by dynamically altering their epigenome.

# 5. Epigenetic Changes Mediated by Salt Stress are Associated with Transcriptional Changes of Stress Responsive Genes

## 5.1 Introduction

### 5.1.1 Salinity Tolerance during Germination and Seedling Growth

Plant's tolerance to salt stress continually changes throughout the developmental stages. Most plants can tolerate salinity at germination stage, but during seedling emergence and early stage of seedling development they become more susceptible to salinity. For example, Maas et al., (1983) reported that maize cultivars were more tolerant to salt stress during germination, but become more sensitive to stress during seedling emergence. Tolerance threshold to salt is higher when plant reached adult stage. Mass and Poss (1989) reported that adult maize and wheat were less sensitive to salt compared to plants at seedling stage (Mass and Poss, 1989).

Salt stress was known to cause delayed seed germination and reduced survival of the seedling (Deinlein et al., 2014). High salinity in plants leads to both ionic (chemical) and osmotic (physical) stress. Ionic stress is mainly caused by excessive intracellular $Na^+$ accumulation, which can induce deficiency of essential ions such as $K^+$, affecting protein synthesis and/or conformation. To cope with $Na^+$ toxicity, plants actively exclude $Na^+$ from cells using ion transporters and regulate the compartmentalization of $Na^+$ into vacuoles. In addition, salt stress also induced osmotic stress that causes reduced water uptake and dehydration. The reduction in germination under salt stress could be attributed to the increased osmotic pressure surrounding the seeds, which affecting water absorption rate and lead to dehydration. The ionic imbalance and accumulation of toxic ions in the seeds may also affect embryos survival and seed germination (Deinlein et al., 2014; Munns and Tester, 2008).

Salt stress is known to induce abscisic acid (ABA) accumulation inside the seed, which then inhibit germination. It has been reported that under salt stress, mutant deficient in ABA biosynthesis are less inhibited during germination. Accordingly, several mutants identified for enhanced germination under high salinity are found to be associated with ABA biosynthesis or signaling pathway (Zhou et al., 2012). However, after germination, mutant deficient in ABA are known to be more sensitive to salt stress, because ABA is required for the activation and regulation of salt stress signaling pathway (Nakashima et al., 2009).

ABA played a pivotal role in regulating the activity of ion transporter that compartmentalize $Na^+$ into vacuoles and exclude $Na^+$ from the cell. In Arabidopsis, sequestration of $Na^+$ within vacuoles is performed by tonoplast-localized $Na^+/H^+$ antiporter 1 (AtNHX1) and NHX1 expression is upregulated by ABA. ABA-mediated compartmentation of $Na^+$ into the vacuoles balances cellular pH homeostasis, maintains turgor pressure and reduces cellular water loss during salt exposure (Gaxiola et al., 1999). AtNHX1 is also known to be involved in $K^+$ compartmentation, overexpression of AtNHX1 in tomato results in higher vacuolar $K^+$ level and increased $K^+$ transport from root to shoot leading to an increase in intracellular $K^+/Na^+$ ratio (Leidi et al., 2010). ABA is also known to interact with the salt overly sensitive (SOS) pathway, an ion transporter that excludes $Na^+$ from cell. Salt stress lead to higher level of $Ca^{2+}$ in cytoplasm, an increase in cytosolic $Ca^{2+}$ is recognized by SOS3, a $Ca^{2+}$ binding protein, which then interacts with and activate a Ser/Thr kinase called SOS2. Calcium and SOS3 activated SOS2 then induces phosphorylation and activation of SOS1, a $Na^+/H^+$ antiporter that exports $Na^+$ out of the cell. Type 2C serine/threonine protein phosphatases ABA INSENSITIVE 2

(ABI2), a negative regulator of ABA signal transduction is known to interacts with and negatively regulate SOS2 activity (Ji et al., 2013).

In summary, the salinity tolerance during germination and seedling growth are regulated by ABA availability and activity in the seeds. ABA biosynthesis and signaling pathway are regulated by networks of genes and transcription factors (TFs). In Arabidopsis, one of the TF families' known to be involved in ABA signaling is MYB (Deinlein et al., 2014). More than 100 MYBs have been identified and many of them are involved in responses to biotic and abiotic stress response. For example, in response to salt stress AtMYB2 are known to interact with calmodulin to activate various salt responsive genes, over-expression of AtMYB44 enhances ABA-regulated stomatal closure and improving plant's tolerance to salt stress, while over-expression of AtMYB15 could increase the expression of genes that involves in ABA biosynthesis and enhance tolerance to salt stress ( Deinlein et al., 2014; Yoo et al., 2005). It was recently reported in Arabidopsis that under salt stress MYB20 could bind to and inhibit the expression of ABI1 and ABI2, a negative regulator of ABA signaling pathway. Since ABA is a positive regulator of salt responsive genes, MYB20 mediated repression of ABI1 and ABI2 might have positive effect on plant tolerance to salt stress. Accordingly, over-expression of MYB20 result in enhanced tolerance to salt stress (Cui et al., 2013).

Following salt stress, ABA-responsive transcription factor could activate various salt-responsive genes. Among early-activated genes, AtCLO3 (ARABIDOPSIS THALIANA CALEOSIN 3, a calcium binding protein) appear to be one of the most highly expressed genes (Kim et al., 2011). AtCLO3 belongs to caleosin protein family that mainly expressed during seed maturation and germination. AtCLO3 is mainly involved in degradation and storage of lipids during embryo development

and germination. Nevertheless, high AtCLO3 expression following ABA, salt and drought treatment suggest that AtCLO3 might also involves in ABA-mediated stress response. In accordance, knockdown mutation of AtCLO3 result in increased stomatal opening and reduced level of drought and salt tolerance (Kim et al., 2011).

Targeted protein degradation by ubiquitin–proteasome system (UPS) also has been suggested to be involved in ABA-salt stress response. In *Arabidopsis* the transcript level of several genes belongs to UPS pathway are upregulated following salt, drought and ABA treatments. It has been proposed that UPS are required to regulate the abundance of various ABA-responsive transcription factors, such as ABI3, ABI and ABI5 (Zhou et al., 2012). Knockdown mutation of genes belong to this pathway, for examples UBIQUITIN-SPECIFIC PROTEASE16 (UBP16) and CNI1 (CARBON/NITROGEN INSENSITIVE 1, an ubiquitin ligase) result in hypersensitivity to salt and higher accumulation of $Na^+$ in leaves (Peng et al., 2014), suggesting their role as positive regulator of salt stress.

### 5.1.2 The Impact of Stress-induced DNA Methylation Dynamics on Gene Expression

Epigenetic marks in form of chromatin modifications and DNA methylation have been proposed to contribute to plants response and adaptation to environmental stress. Epigenetic changes associated with stress have been traditionally associated with the transcriptional regulation of stress-related genes and enhanced tolerance against stresses (Kinoshita and Seki, 2014; Mirouze and Paszkowski, 2011). *Arabidopsis* plants that were pretreated with mild salt stress at the seedling stage followed by growth under non-stress conditions display better tolerance to salt stress

during adult stage that non-pretreated plants. This increased stress tolerance was associated with a change in H3K27me3, primarily at transcriptional regulators (Sani et al., 2013). Intriguingly, one of the genes that displayed a loss in H3K27me3 in the pretreated plants is AtHKT1 that was found to be upregulated and could explain the increased tolerance observed. A separate study found that the promoter of AtHKT1 contains tandem repeats that produce smRNAs which directs non-CG methylation onto this region leading to a reduced AtHKT1 expression in leaves. Deletion of this promoter region results in hypersensitivity to salt stress suggesting that the epigenetic state of this region is important for tissue-specific transcriptional regulation and salt tolerance (Baek et al., 2011). Similarly, the transcription of AtMYB74 (member of MYBs transcription factors family) under salt stress has been found to be epigenetically regulated by non-CG methylation. Epigenetic regulation of this gene by RdDM is essential for salt tolerance during germinaton (Xu et al., 2015). In soybean (*Glycine max*), salt stress affects the methylation state of promoter and coding region of four salt-responsive transcription factors (one *MYB*, one *b-ZIP* and two *AP2/DREB* family members). Treatment with the DNA methylation inhibitor *5-aza-2-deoxycytidine* caused upregulation of these four transcription factors, suggesting that their expression was regulated by the methylation status of its promoter (Song et al., 2012). In a separate study using wheatgrass (Thinopyrum ponticum) salt stress was found to induce hypomethylation in the promoter of twenty four salt responsive genes. Loss of promoter methylation after salt stress or 5-azaC treatment was accompanied with enhanced gene expression (Wang et al., 2014).

Stress induced DNA methylation changes that are associated with transcriptional changes not only occurr in genic regions. Exposure to stress could lead to DNA

methylation changes at intergenic regions or Transposable Elements (TEs) and affect the expression of nearby genes. Dowen et al. (2012) showed that biotic stress lead to global genome methylation changes in TEs located downstream or upstream of protein coding genes. These genes were enriched for pathogen defense gene and transcriptional regulator. Demethylation of TEs following biotic stress was correlated with increased expression of TEs and smRNAs production that in some cases caused upregulation of proximal genes (Dowen et al., 2012). TEs are also epigenetically targeted by heat stress. Popova et al. (2013) found that the majority of protein coding genes miss-regulated in mutants defective in RdDM following heat stress are located in proximity to TEs, suggesting that transcriptional regulation of heat responsive genes during stress involved methylation changes in TEs (Popova et al., 2013). Similarly, Ito et al. (2011) showed that during heat stress, RdDM mediated retrotransposition of heat responsive *COPIA*-type retroelement ONSEN could result in the miss-regulation of genes adjacent to ONSEN new insertion site (Ito et al., 2011). In rice, insertion of a TE named mPING was associated with the cold-responsive expression of nearby genes (Yasuda et al., 2013). Recently, Makarevitch *et al.* (2015) showed that following abiotic stress treatment, small number of maize TEs families could be implicated in the transcriptional regulation of adjacent genes by providing stress-responsive enhancer-like functions. Insertion of TEs into protein-coding genes might provide a binding sites for transcription factors or influencing chromatin packaging, thus providing regulatory variation in gene expression (Makarevitch et al., 2015). In summary, environmental stress could induce methylation changes in genes or TEs adjacent to genes and in some instanced this stress-induced methylation change could alter the transcriptional of stress related genes thus providing plants with tools to better tolerate stress.

### 5.1.3 Chapter Aims

To evaluate whether salt-induced DNA methylation changes are targeted to genes or regions of the genome associated with the transcriptional regulation of salt responsive genes and with an enhanced tolerance to high salinity

## 5.2 Results

### 5.2.1 Epimutations Induced by Salt Stress Map Near Genes Implicated in Stress Tolerance

In the previous chapter I have shown that multigenerational salt stress treatments induce hypo- or hypermethylation primarily at TEs or intergenic sequences flanking genes. I found total of 446 genes that were associated with salt-induced DMRs. To evaluate whether genes associated with DMRs are enriched in a particular molecular or biological function I performed Gene Ontology (GO) analysis. Notably, DMRs-associated genes showed a statistically significant enrichment for genes related to salt stress response, such as electron and anion transport, response to stress, protein folding, calmodulin binding and ion channel activity. However, none of these categories were consistently enriched in all generations (Figure 19). When comparing salt-induced DMRs that overlap or do not overlap with spontaneous mutation accumulation DMRs (MA-DMRs), both MA Overlap/Non-overlap DMRs showed an enrichment for GO related to stress reponses such as ion transport and calmodulin binding, suggesting that both set of DMRs were not fundamentally different (Figure 19). GO analysis also revealed that genes in proximity with hypermethylated DMRs were primarily associated with response to stress and metabolic process. On the other hand, genes in proximity to hypomethylated DMRs were associated with RNA-directed DNA polymerases, reverse transcriptases, and methyltransferase activity. Results from GO analysis suggest that hypermethylation are targeted to regions that regulate general stress responses, while regions that involved in epigenetic regulation undergo hypomethylation

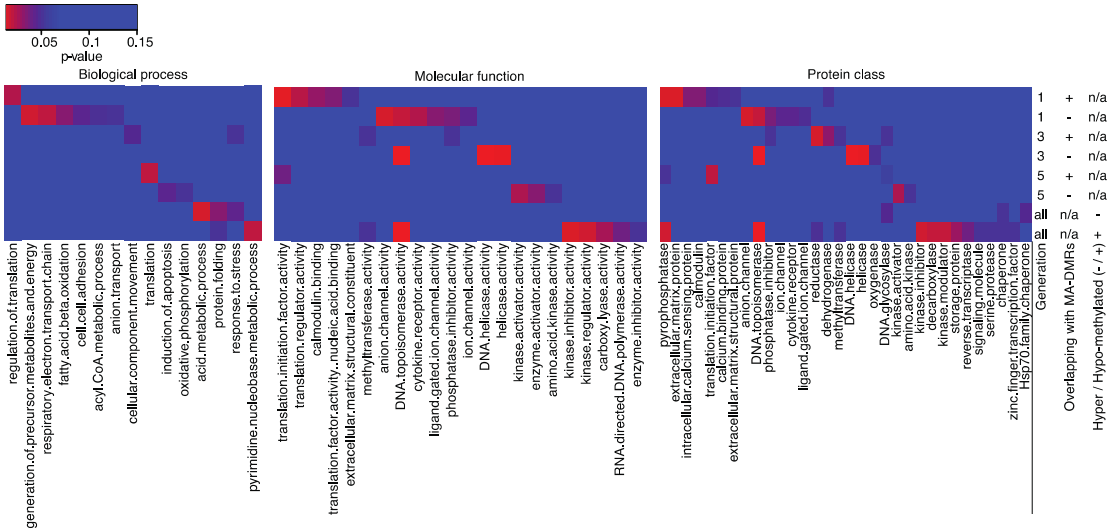


**Figure 19. Gene Ontology analysis of genes near salt induced-DMRs.** Heatmap showing significantly enriched GO categories between MA Overlap/Non-overlap and hypo-/hypermethylated DMRs (p-value < 0.05). Colours toward red indicate low p-values, violet indicates marginal p-values, and blue indicates no statistically significant difference.

To further validate that salt-induced methylation changes are targeted to regions proximal stress-related genes, I used publicly available microarray data to analyze the effect of salt stress on the transcription of genes associated with salt DMRs. From the 446 genes identified, 123 genes (27.58%) are differentially expressed in response to salt stress (displaying more than 1.8 fold expression change in response to salt treatment, in root and/or shoot) (full list of genes can be found in Appendix Table 7). A subset of these genes such as: MYB20 (MYB DOMAIN PROTEIN 20, a transcription factor) (Cui et al., 2013), CNI1 (CARBON/NITROGEN INSENSITIVE 1, an ubiquitin ligase) (Peng et al., 2014), and ATCLO3 (ARABIDOPSIS THALIANA CALEOSIN 3, a calcium binding protein) (Kim et al., 2011) are known to be involved in salt stress tolerance (Figure 20).

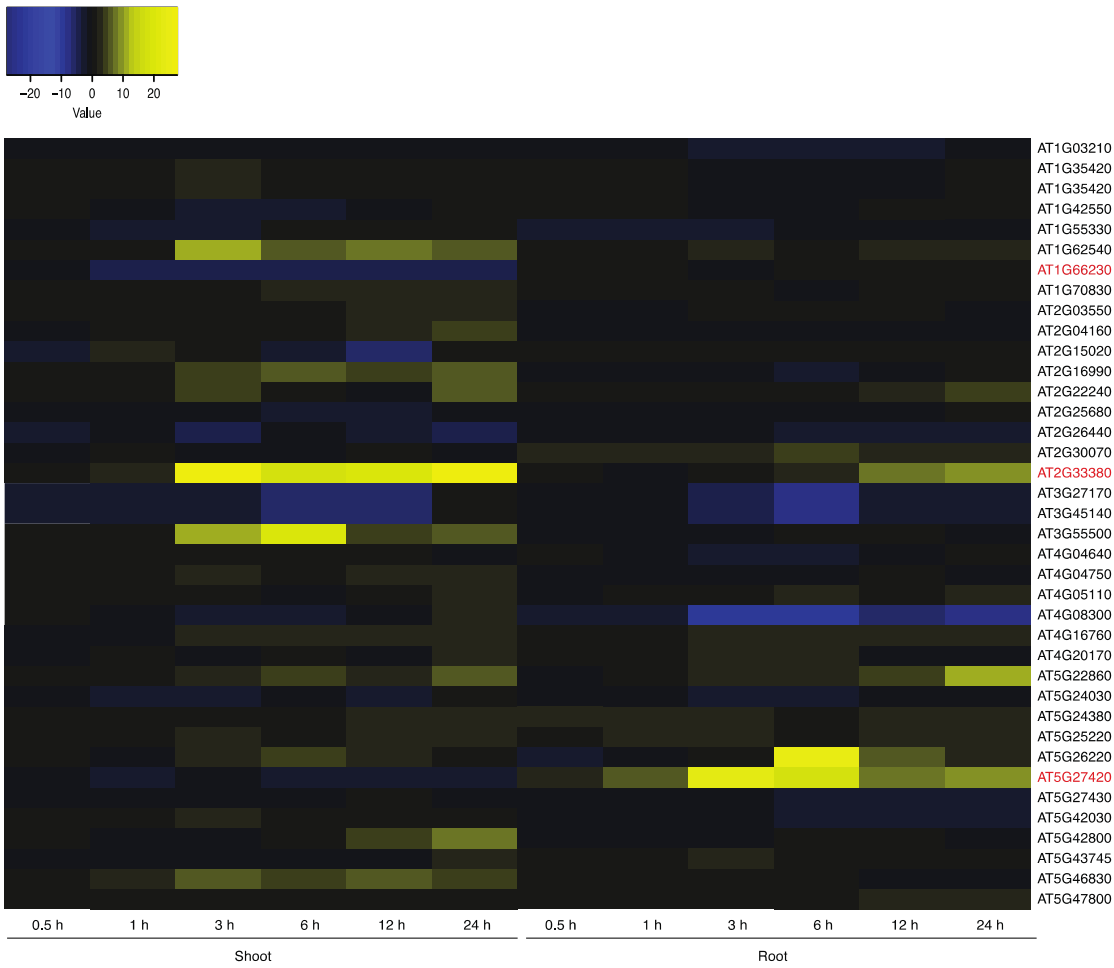


**Figure 20. Expression of DMRs-associated genes under salt stress conditions.** Heatmap showing expression in *Arabidopsis* root or shoot following exposure to 150 mM NaCl for 0.5h, 1h, 3h, 6h, 12h or 24h. Colours toward yellow indicate up-regulation after stress, blue indicates down-regulation after stress, and black indicate no changes in expression after stress. The expression data was collected from publicly available microarray data (www.expressionbrowser.com).

### 5.2.2 Salt Stress Induced Epimutation are Associated with Intergeneic Long non-coding RNAs (lncRNAs)

Several studies have suggested that stress-induced DNA methylation changes are linked to changes in gene expression. To investigate whether salt induced DMRs are truly affecting the transcriptional response of nearby genes to salt stress, I performed an expression analysis by a quantitative Real-Time PCR (qRT-PCR) on genes associated to salt-DMRs and known to be involved in salt stress response. One the gene tested was MYB20, a transcription factor that is involved in abscisic acid (ABA) signaling and has been implicated in stress tolerance (Cui et al., 2013). The MYB20-associated DMRs is located in a TE sequence 1.3kb upstream of transcription start site. In response to salt stress this region is hypermethylated in PO and P1 plants, but being reset to control level in the P2 (Figure 21A). I found that the expression of MYB20 is downregulated in progenies of salt stressed plants compared to the progenies of control plants. MYB20 is downregulated when grown on normal salt-free condition and on media with high salinity, both in P1 and P2 generations. (Figure 21B). Another gene tested was *CNI1,* a membrane RING-type ubiquitin ligase implicated in metabolic sensing and genetic lesions on this gene confer hypersensitivity to salt (Peng et al., 2014). The salt-DMR associated with this gene is located inside a TE located downstream of the TSS. Following multigenerational salt stress treatment this region becomes hypomethylated in the stressed-PO plants and in the P1 and P2 progenies of stressed plants (Figure 22A). There are no different in CNI1 expression between the progenies of stressed and non-stressed plants when the plants were grown under normal salt-free condition. However, high salinity led to a strong reduction in CNI1 expression in progeny of untreated plants, with substantially attenuated reduction in the progeny of stressed plants. The CNI1

transcriptional response to salt stress was back to normal level in the P2 (Figure 21B). The third gene tested was ATCLO3 (ARABIDOPSIS THALIANA CALEOSIN 3, AT2G33380). This gene has 2 splice variant called AT2G33380.1 and AT2G33380.2 and it encodes a calcium binding protein (Kim et al., 2011). The salt-DMR associated with ATCLO3 is in the gene body (Figure 21A). When plants were grown under normal conditions the progenies of stressed and non-stressed plants showed no differences in transcription of the two splice forms. However, under salt stress, the expression of both splice variants was significantly reduced in P1 progenies of stressed-plants compared to the progenies of control plants. The expression of both ATCLO3 splice variant went back to normal levels in the P2 (Figure 21B).

Because I found that the transgenerational phenotypic responses to salt tress are impaired in various epigenetic mutants, I tested whether the transcriptional response to salt stress is also affected in these mutants. The progenies of stressed and non-stressed plants were both mis-regulated for MYB20 and CNI1 expression in DNA demethylation mutants after exposure to salt. In addition, the transcriptional responses observed for these genes in P1 or P2 progenies was not visible in either *cmt3* or *nrpd1a* mutants. Moreover, the transgenerational response of ATCLO3 to salt was altered in *rdd*, *cmt3* and *nrpd1a* mutants (Figure 21B). Taken together, these data show that the transgenerational transcriptional responses to salt are altered by dynamic DNA methylation changes.

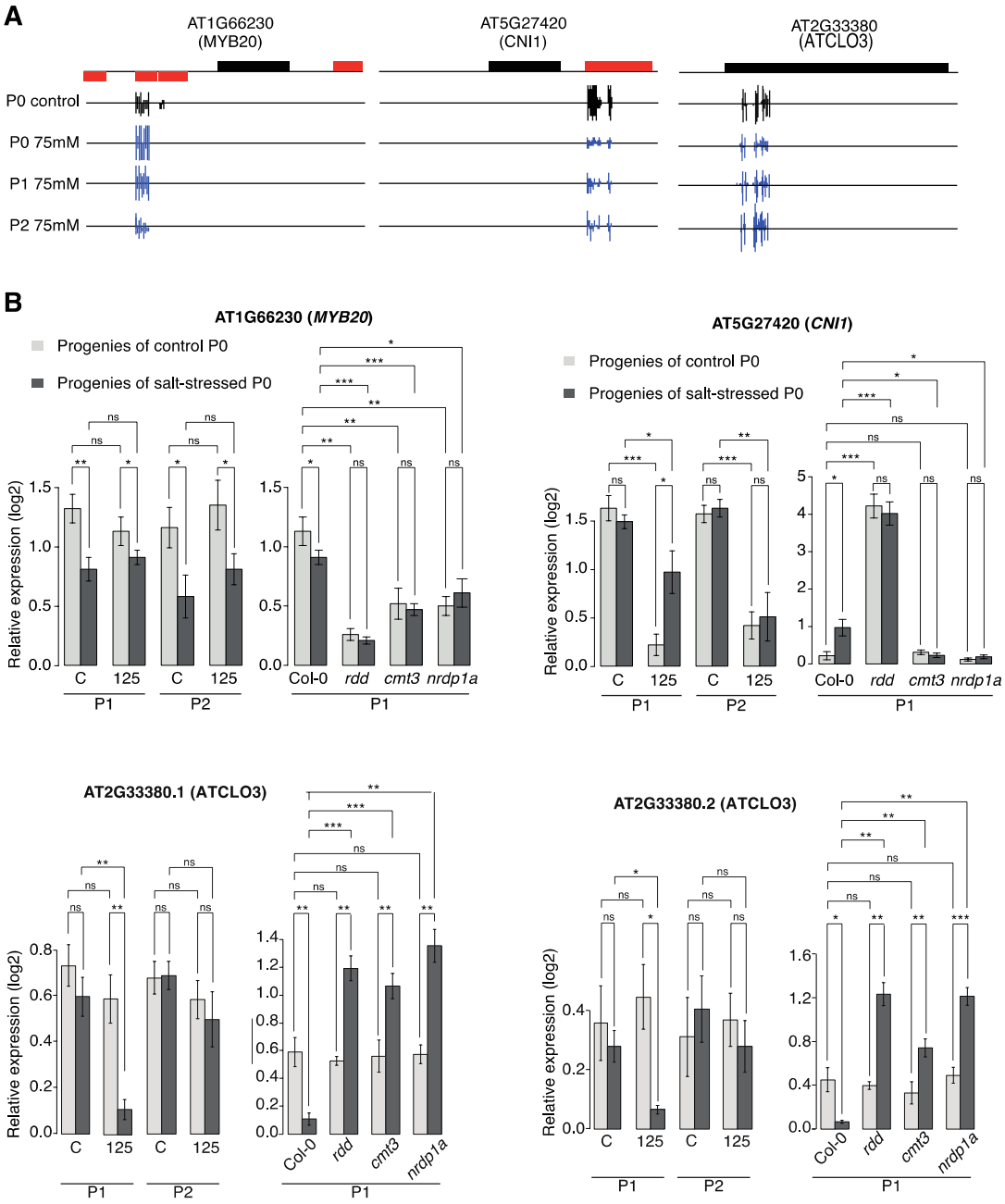


**Figure 22. Transcriptional regulation of salt responsive and DMRs-associated genes.** (A) Genome browser snapshots of DMRs in proximity to *MYB20,* CNI1 and ATCLO3. The top panel is a schematic representation of the respective genomic locus; black boxes represent genes, red boxes represent TEs. Vertical bars indicate methylation at the respective DMR in the control P0 (black), and the salt-treated P0 and its progeny (blue). (B-C) Expression of genes shown in (A) in the P1 and P2 progeny of non-stressed control and salt-stressed P0 plants, in wild type and mutants. Quantitative Real-Time PCR was performed on RNA extracted from leaves of 2-week-old plants grown on MS or MS supplemented with 125 mM NaCl (left panel) or only on 125 mM NaCl (right panel). Wild-type samples in the right panel correspond to 125 mM NaCl samples in the left panel and are repeated for clarity. Asterisks indicate significant differences relative to controls (unpaired Student's *t*-test; ** $p<0.01$; * $p<0.05$; NS $p>0.05$). Error bars indicate standard deviation. ("C"=control; "125"=125 mM NaCl).

Salt stress not only affects the expression of protein-coding genes but it is known to also affect the expression of long noncoding RNAs (lncRNAs) (Liu et al., 2012; Matsui et al., 2008). Liu et.al (2012) reported that 1832 lncRNAs are differentially expressed following 2 hours and/or 10 hours of drought, cold, salt and/or ABA treatments, and they suggested that lncRNAs could play a fundamental role in plant response to stress. To investigate the possible connection between salt-induced DMRs and stress responsive lncRNAs, I assessed how many DMRs-associated genes were enriched for stress-responsive antisense lncRNAs (Fisher's exact test, p= 0.008). I found that 45.52% of genes with salt-stress DMRs were in direct overlap with stress-responsive antisense lncRNAs. For example, CNI1 has an antisense lncRNA that it is rapidly upregulated when exposed to salt (Figure 22). This finding suggests that salt-induced DNA methylation changes not only affect coding-gene expression but also antisense lncRNA expressin. To evaluate whether salt induced DMRs are truly affecting antisense lncRNAs expression, I focused my analysis on CNI1. I found that there was no significant differences in CNI1-lncRNA expression when comparing progenies of stressed and non-stressed plants grown under normal condition. However, following salt stress exposure the expression of CNI1-lncRNA was up-regulated in the progeny of non-stressed control plants but down-regulated in the P1 progeny of stressed plants. This differential expression was retained over two generations in the absence of stress (Figure 23). Intriguingly, salt mediated CNI1 coding-gene and antisense lncRNAs was anti-correlated thus indicating that lncRNAs might act as negative regulator of CNI1 expression. Further, the transcriptional response of CNI1 antisense lncRNA was impaired in DNA demethylation mutants thus adding to the role of the identified salt-DMR in the regulation of this gene.

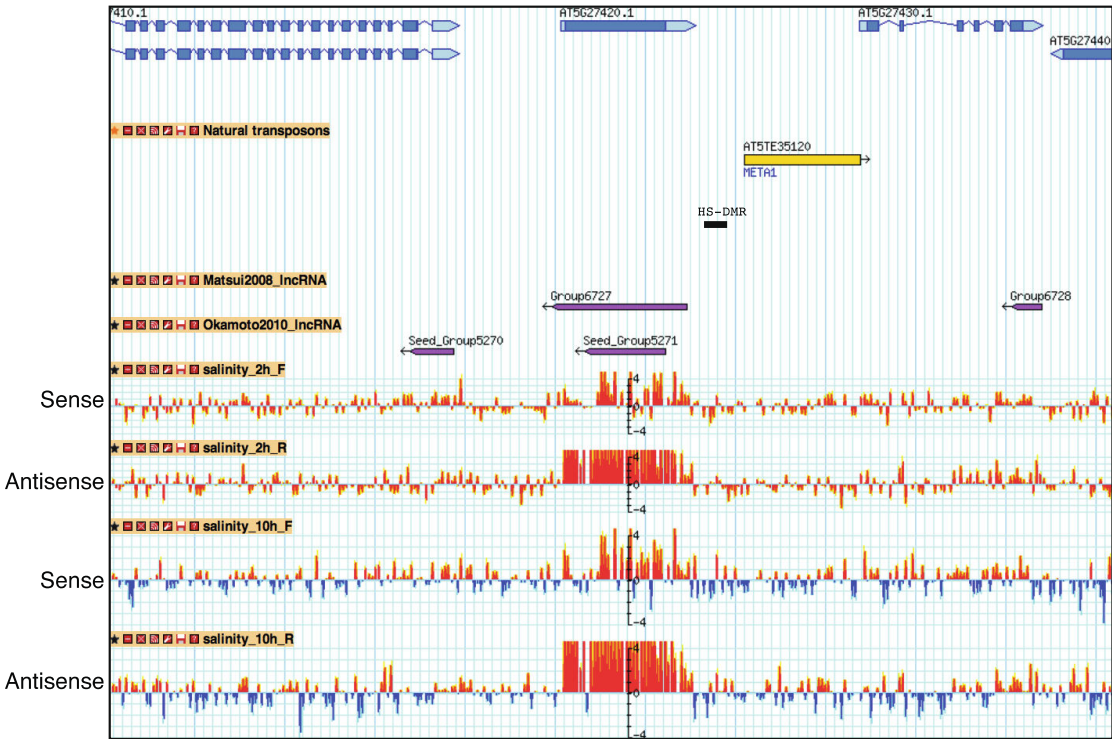


**Figure 22. Genome browser view of the genomic region flanking CNI1.** Tracks represent gene annotations (blue), transposons (yellow) and HS-DMR (black), lncRNAs (purple) and signal of tilling array hybridized with labelled RNA extracted from plants exposed to high salinity for 2 and 10 hours (red and blue bars). Signal corresponding to sense and antisense strands are indicated (Jin et al. 2013).

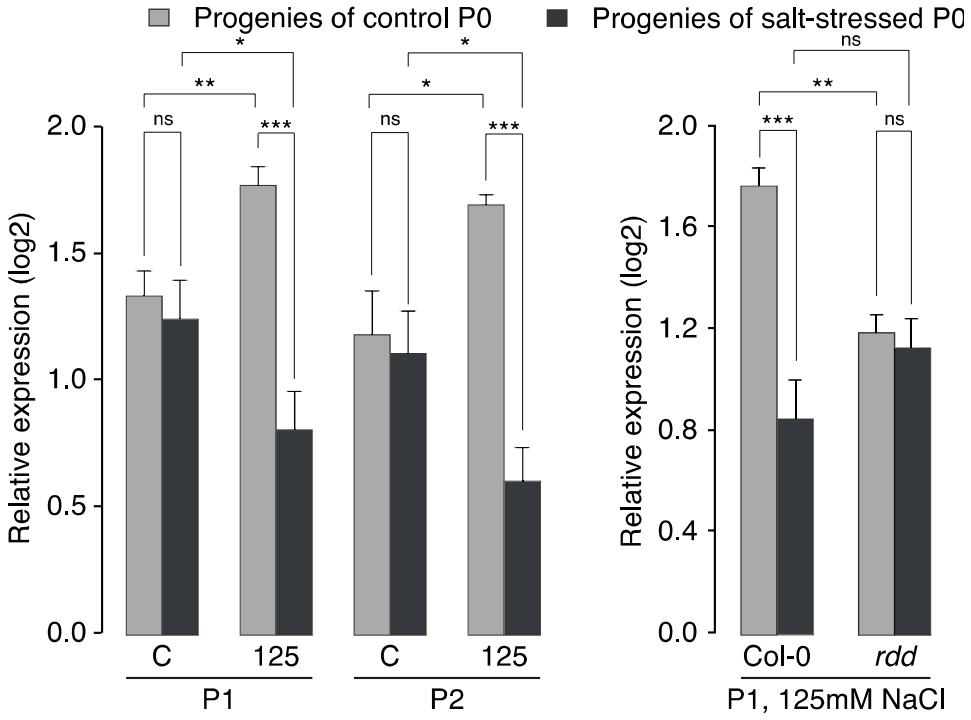


**Figure 23. Expression analysis of of CNI1 antisense lncRNA in naïve and primed plants.** Expression of CNI1 antisense lncRNA genes shown in the P1 and P2 progeny of non-stressed control and salt-stressed P0 plants, in wild type and mutants. Quantitative Real-Time PCR was performed on RNA extracted from leaves of 2-week-old plants grown on MS or MS supplemented with 125 mM NaCl (left panel) or only on 125mM NaCl (right panel). Wild-type samples in the right panel correspond to 125 mM NaCl samples in the left panel and are repeated for clarity. Asterisks indicate significant differences relative to controls (unpaired Student's *t*-test; ** $p<0.01$; * $p<0.05$; NS $p>0.05$). Error bars indicate standard deviation. ("C"=control; "125"=125 mM NaCl).

To confirm whether the salt-induced DMRs are truly responsible for regulating the expression of stress-responsive genes and antisense lncRNAs during stress, I performed expression analyses on two independent T-DNA insertion lines, named cni1-2 (SALK_100221) and cni1-3 (SALK_030235), harboring a ~4Kb T-DNA insertion between CNI1 and the identified salt-induced DMR (Figure 24A). I found that the expression of CNI1 after salt exposure was significantly mis-regulated in both insertion lines when compared to wild-type plants (Figure 24B). Further, expression analysis also showed that compared to wild-type, both insertion lines

were impaired in the transcriptional stress response of CNI1 antisense lncRNA (Figure 25C). Taken together, these data indicates that salt-induced DMRs could act as short-distance regulatory elements for coding and lncRNA transcription.

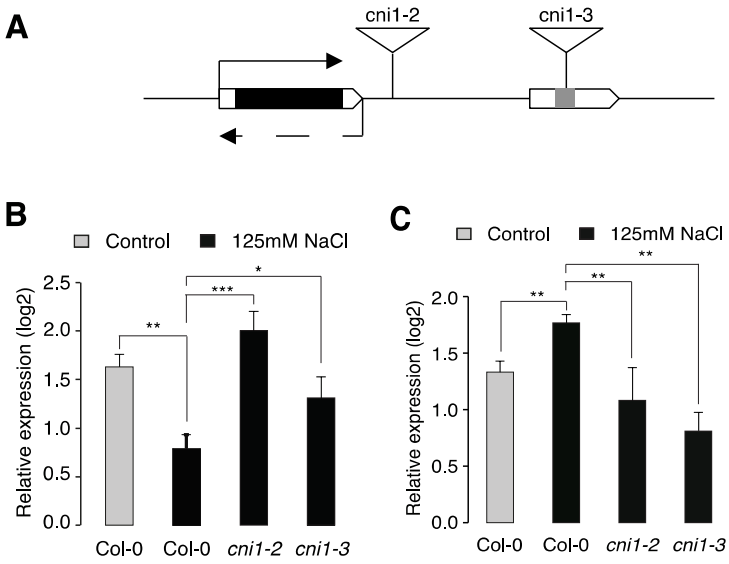


**Figure 24. Trancriptional regulation of CNI1 sense and antisense transcripts by distant acting salt-induced DMRs.** (A) Schematic diagram of the CNI1 locus highlighting; coding transcript (solid arrow line), antisense lncRNA transcript (dashed arrow line), transposable element with HS- DMR (white and grey box respectively), transgenic insertion elements (triangles) (B) Analysis of CNI1 coding-gene expression following salt stress treatment. (C) Analysis of lncRNAs expression after exposure to hyperosmotic stress. Quantitative Real-Time PCR was performed on RNA extracted from leaves of 2-week-old plants grown on MS (grey bar) or MS supplemented with 125 mM NaCl (black bar). Samples analysed: wild type, cni-2 (SALK_100221) and cni-3 plants (SALK_030235).

## 5.3 Discussion

Plants use complex mechanisms to cope with ionic and osmotic stress caused by high-salinity. In this chapter I have shown that some salt-responsive genes are modulated by DNA methylation dynamics. Many of the salt-induced DMRs identified are located at TEs or intergenic regions proximal to protein-coding genes. The dynamic changes in TE methylation partially correlate with changes in expression of adjacent genes thus suggesting that the expression of some stress-responsive genes is under the control of short-range regulatory elements that epigenetically modulated in response to environmental stimuli. Some salt-induced DMRs, such is the case for CNI1-DMR, can be stably transmitted over several generations in the absence of stress, which could enable plants with a better tolerance against salt stress. The expression of key stress-response regulators and tolerance to salt stress are both affected in DNA methylation and demethylation mutants, thus supporting the model that DNA methylation can change dynamically in response to environmental stimuli to alter gene expression through short-range chromatin interactions. For many plant species, TEs and intergenic regions comprise the majority of plants genome TEs and intergenic regions have been traditionally considered a mutagenic, deleterious, or neutral component of the genome. However, my work provides further evidence for the interplay between TEs and stress-responsive expression and adaptation. The exact mechanism(s) implicated are still unclear but these sequences could have enhancer-like effects (Ito et al., 2011; Makarevitch et al., 2015). It is likely that the dynamic transcriptional regulation of key stress-reponsive genes is mediated by the action of antisense lncRNAs. This could explain why a significant portion of lncRNAs is dynamically expressed in response to stress. The mechanism by which antisense lncRNAs can regulate sense

transcription is not well understood but interaction with proteins that regulate transcription, translation or affecting mRNA stability has been proposed (Bardou et al., 2014; Heo and Sung, 2011; Swiezewski et al., 2009). Future work will be required to determine the precise relationship between DNA methylation, TEs, genes and antisense lncRNAs.

### 5.4 Summary

My data suggest DNA methylation changes mediated by salt-induced alter the transcriptional regulation of salt-responsive genes. Plants able to inherit these epigenetic changes are able to display enhanced tolerance to stress.

# 6. General Discussion

## 6.1 Adaptive Responses Acquired During Recurrent Exposure to Environmental Stress

Our current understanding of evolution is based on the modern evolutionary synthesis, also known as "neo-Darwinism". This paradigm assumes that natural selection acts solely on phenotypes determined by DNA sequence variation within natural populations (Nei, 2005). However, in recent years, scientists have revealed that epigenetic information on the form of DNA methylation can be stably inherited across multiple generations. Several reports have suggested that these heritable epigenetics changes have extensive morphological and physiological effects (Kinoshita and Seki, 2014; Mirouze and Paszkowski, 2011). These new findings imply that epigenetic inheritance could be an additional factor implicated in evolution. However, it is yet unclear whether environment events perceived by plants can induce epigenetic changes that can be inherited and have significant ecological consequences at the population level.

To be able to address this unresolved questions, I designed a systematic approach to evaluate the impact of epigenetic adaptation in plants following multigenerational stress treatment. Salt stress by sodium chloride (NaCl) application has been previously shown to generate enhanced tolerance to stress (Boyko et al., 2010). My data has revealed that exposure to salt stress in a single generation is not sufficient to induce noticeable phenotypes to the offspring. Contrary to current views, my data supports the view that recurrent exposure to salt stress, over 2 or more generations, is strictly required to induce transgenerational effect manifested as an increased tolerance to high salinity in the direct progeny. My data also shows that this acquired tolerance is rapidly lost in subsequent generations in the absence of stress. This finding suggests that plants are unable to generate a stable stress memory in the

absence of frequent environmental cues. Even when plants have been repeatedly exposed to salt stress for five consecutive generations, the acquired tolerance is rapidly lost in the second generation in the absence of stress. Although several studies have reported that adaptive responses to environmental stresses can be transmitted across many generations in the absence of stress (Luna et al., 2012; Rasmann et al., 2012; Slaughter et al., 2012) the biological benefits of such phenomenon has been questioned. Activation of the stress tolerance pathway is often accompanied with fitness costs in the form of repressed growth and development (Denance et al., 2013; Denby and Gehring, 2005). Because plants are sessile organism that are constantly exposed to an ever-changing environment, stress response mechanisms to stress must be sensitive yet reversible. Therefore, it is important for plants to reset stress-mediated responses and return to a normal state once stress is relieved. If plants could rapidly accumulate stable epimutations in response to stress, they could results in significant fitness costs. An evolutionary strategy based in the accumulation of environmentally acquired epimutations would be unfavourable for the survival of individuals within a population. For this reason epigenetic and adaptive responses to stress theoretically need to be reversible. This reversible response would benefit parental plants when exposed to stress, but not affecting the fitness of offspring in the absence of stress. The balance between benefit and fitness cost could favour stable epimutations only when stress conditions persist over multiple generations.

Several studies have repeatedly proposed that transgenerational epigenetic inheritance could influence evolution in a number of organisms, including plants (Heard and Martienssen, 2014; Mirouze and Paszkowski, 2011). However, my data questions the long-term evolutionary impact of environmentally induced epigenetic

inheritance. Instead, my data indicates that plants posses a short-term epigenetic memory that allow them to rapidly adapt to environmental fluctuations.

### 6.2 Dynamic Regulation of DNA Methylation in Response to Stress

Several studies have reported that environmental stress can induce genome-wide changes in DNA methylation (Bilichak et al., 2012; Jiang et al., 2014). My data however, does not support this hypothesis. Instead, I have found that salt stress leads to small but targeted DNA methylation changes.

Transgenerational phenotypic and transcriptional effect observed in the P1 of salt-stressed plants were absent in mutants defective in *de novo* DNA methylation and demethylation pathyways, which are known to regulate the activity of transposable-elements (TEs). These data suggest that an intergenerational response to salt stress involves regulation of TEs methylation. In support of this hypothesis, I found that discrete methylation changes in response to salt were particularly targeted to non-CG sites in transposable-elements (TEs) sequences. The exact mechanism of how salt stress could activate *de novo* DNA methylation and demethylation pathways to alter the epigenetic state of specific sequences in the plant genome is still unclear. Salt stress is known to trigger higher level of $Ca^{2+}$ in the cytoplasm and induces accumulation of various secondary messengers such as reactive oxygen species (ROS), nitric oxid, and hormones (Jiang et al., 2013). Accumulation of these secondary messengers activates a cascade of kinase signaling pathways and downstream of these signaling cascades various transcription factor families are differentially expressed (Golldack et al., 2014). It is likely that some of these stress-responsive signaling cascades are involved in the regulation of *de novo* DNA

methylation and demethylation pathways. Recent studies have shown that RNA splicing factors could act as intermediate components between stress signalling and epigenetic responses. For example, splicing factors SR45 (Ausin et al., 2012), ZOP1 (Zhang et al., 2013), STA1 (Dou et al., 2013), and PRP31 (Du et al., 2015) affect Pol IV-dependent small RNA accumulation, an integral component in *de novo* DNA methylation through the RNA dependent DNA methylation (RdDM) pathway. Intriguingly, the expression of these splicing factors is modulated by various plant hormones and environmental stresses and genetic lesions display hypersensitivity to salt stress during germination (Huang et al., 2013). However how these splicing factors affect RdDM-dependent DNA methylation still remains unclear.

Stress-induced reactive oxygen species (ROS) could also act as a regulatory link between stress signaling and epigenetic machinery. Mechanical wounding of maize leaves leads to a transient global decrease in DNA methylation level. This global hypomethylation (20-30% decrease in DNA methylation) occurred one hour after treatment, and reverted back to the basal in the next hour. Interestingly, wounding also caused a two-step ROS accumulation. First, ROS production increased rapidly one minute after wounding and decreased significantly to initial levels between half to one hour after wounding. Intracellular ROS level increased two hours after wounding but reverted back to basal level after four hours (Lewandowska-Gnatowska et al., 2014). The wounding-induced hypomethylation was associated with the up-regulation of a stress-responsive calcium-dependent protein kinase *ZmCPK1* (Lewandowska-Gnatowska et al., 2014). Choi and Sano (2007) reported that tobacco plants exposed to paraquat, an effective ROS generator, display a significant decrease in DNA methylation one hour after treatment. Paraquat treatment also caused the induction of stress-responsive glycerophosphodiesterase-

like protein NtGPDL, which is associated with DNA demethylation (Choi and Sano, 2007). These studies suggest that ROS may be involved in the regulation of DNA methylation dynamics in plants. The ROS signaling pathway is known to play a critical role in stress tolerance (Choudhury et al., 2013). However, the precise mechanism(s) implicated in the regulation of DNA methylation by ROS accumulation remains to be elucidated.

### 6.3 Epigenetic Priming of Naïve Plants Following Stress

My work has revealed that repeated exposure to stress over two or more generations is able to induce improved tolerance to stress in the direct progeny. I also found that this acquired tolerance was lost in the subsequent non-stressed generations. In accordance to the phenotypes observed, I found that salt-induced methylation changes were prominent in plants that were directly exposed to stress, and these changes were gradually erased in subsequent non-stressed progenies. In addition, I found that only a small fraction of DNA methylation changes were transmitted to the immediate progeny and only if parental plants were repeatedly exposed to stress for at least 2 generations. These results indicate that naïve plants (plants that have never been exposed to stress) and primed plants (plants that have already experienced stress) have different epigenetic and physiological responses to stress. This view is supported by the substantial overlap (>30%) found between salt–induced DMRs established in plants exposed to stress over three and five generations (Figure 16). On the other hand, only a small overlap was found between methylation changes in plants exposed to stress for a single generation compared to plants exposed to stress over three or five generations (<5%) (Figure 16).

Moreover, I also found that half of the salt-induced DMRs identified in this study overlapped with spontaneous DMRs found in a near-isogenic populations grown under control environment for 30 generations (Becker et al., 2011). This finding indicates that specific regions of the genome are more susceptible to epigenetic changes. Pre-exposure to stress may alter the epigenetic state of plants and facilitate the establishment of new epigenetic marks at labile genomic regions when repeatedly exposed to stress. This "primed" state might affect their response to subsequent stress, allowing them to generate a stronger and/or faster response. Interestingly, repeated exposure to salt stress over five generations did not significantly increase the number of newly acquired epimutations or produce a stronger adaptive response, thus suggesting that plant phenotypic and epigenetic plasticity is limited to a certain level. Response to environmental stress may be limited to specific regions of the genome, hence the accumulation of environmental epimutations is restricted.

How naïve plants establish "primed" epigenetic states following stress is still unknown. My data shows that salt-induced DNA methylation changes are erased in the progeny of plants exposed to stress for only one generation, implying that other epigenetic marks might retain this "stress memory" in naïve plants. Beside changes in methylation, plants were known to undergo chromatin changes in response to a wide range of stresses (Luo et al., 2012). I found a 35% overlap between salt-induced DMRs and salt-induced changes in H3K27me3 occupancy (Sani et al., 2013), suggesting that methylation and chromatin changes in response to salt stress are targeted to the same discrete regions of genome. It has been shown in mammals that DNA methylation acts globally to antagonize the placement of H3K27me3 (Hagarman et al., 2013). In accordance to this report, I have found that

hypermethylation in response to salt stress is associated with decrease in H3K27me3 occupancy. Salt stress might alter H3K27me3 occupancy in naïve plants and could affect the DNA methylation response in the subsequent stressed generations. Chromatin changes for plant defence-response genes has been found after pathogen attack (Luna et al., 2012), however maintenance of these stress-induced chromatin changes in subsequent generations in the absence of stress have not yet been reported. Future studies should focus on the elucidation of the relationships between DNA methylation and chromatin using genetic lesions for these epigenetic pathways.

### 6.4 Non-equivalent Parental Contribution Acquired Adaptation to Salt Stress

Several studies have attempted to link the novel phenotypes induced by stress and global epigenetic changes (Kinoshita and Seki, 2014; Mirouze and Paszkowski, 2011). My data instead shows that phenotypes changes induced by stress are associated to small changes in DNA methylation. Remarkably, I also found that adaptive responses to stress are not equally transmitted through lineages. This non-equivalent parental contribution could be due to differences in the transmission of salt-induced DNA methylation changes in gametes. It has been previously proposed that during gametogenesis, the plant male germline undergoes extensive DNA methylation reprogramming (Calarco et al., 2012; Ibarra et al., 2012). In accordance to this view, I have found that only a limited amount of stress-associated DNA methylation changes are present in male gametes, thus suggesting that salt-induced methylation changes are actively reset in the male gametes. I found that sperm cells (SC) and companion vegetative cells (VC) were differentially methylated (13,776 DMRs) and that these DMRs were predominantly found in TEs located near genes. Interestingly, I found a highly significant overlap between SC-VC DMRs and salt-

induced-DMRs (Figure 4), suggestive of an active reprogramming in these genome regions during male gametogenesis. I have found that the DNA glyclosylase DEMETER (DME) is implicated in the erasure of stress induced adaptive traits induced by high salinity, which may explain the limited amount of stress-induced DNA methylation marks present in male gametes and the immediate progeny. How DME could selectively target stress-induced DNA methylation marks is currently unknown. Future work will focus in elucidating the precise role of DME in erasing epigenetic information induced by stress.

### 6.5 Stress-induced DNA Methylation Influences the Expression of Adjacent Genes

My data show that DNA methylation changes in response to salt stress are primarily targeted to Transposable Elements (TEs) sequences adjacent to protein coding genes. These stress-induced epigenetic marks are associated with dynamic expression changes in response to stress. Many of the genes implicated are involved in salt stress tolerance, suggesting that tolerance and acclimatization to high salinity are significantly regulated epigenetically. Interestingly, I also found that half of the epigenetically controlled genes identified in this study are in direct overlap with stress-responsive antisense long noncoding RNAs (lncRNAs). Disruption of regulatory TE sequence by T-DNA insertion not only affected the stress responsiveness of nearby genes but also the expression of associated lncRNAs . In plants, antisense lncRNAs have been implicated in modulating mRNA stability and splicing, interaction with transcriptional regulators, and directing chromatin changes (Bardou et al., 2014; Heo and Sung, 2011; Swiezewski et al., 2009). The significance and mechanistic role of the antisense lncRNAs identified in this study is unclear.

One possibility is that lncRNAs could act as a regulatory link between TEs and gene expression under stress conditions. My data from CNI1 transcriptional analysis, support a model where plants that are grown under normal non-stress conditions have TE sequences in a hypermethylated state, mediated by the RdDM pathway, that have minor effects on the stress-mediated response of adjacent genes. However, when plants have been exposed to stress, DNA demethylation is actively removed from TE sequences that then could act as short-range transcriptional regulators of antisense lncRNAs and result in the stress-mediated repression of associated genes. Under this model, the disruption of the regulatory effects imposed by TE sequences will lead in a differential response to stress of naïve and primed plants (Figure 25).

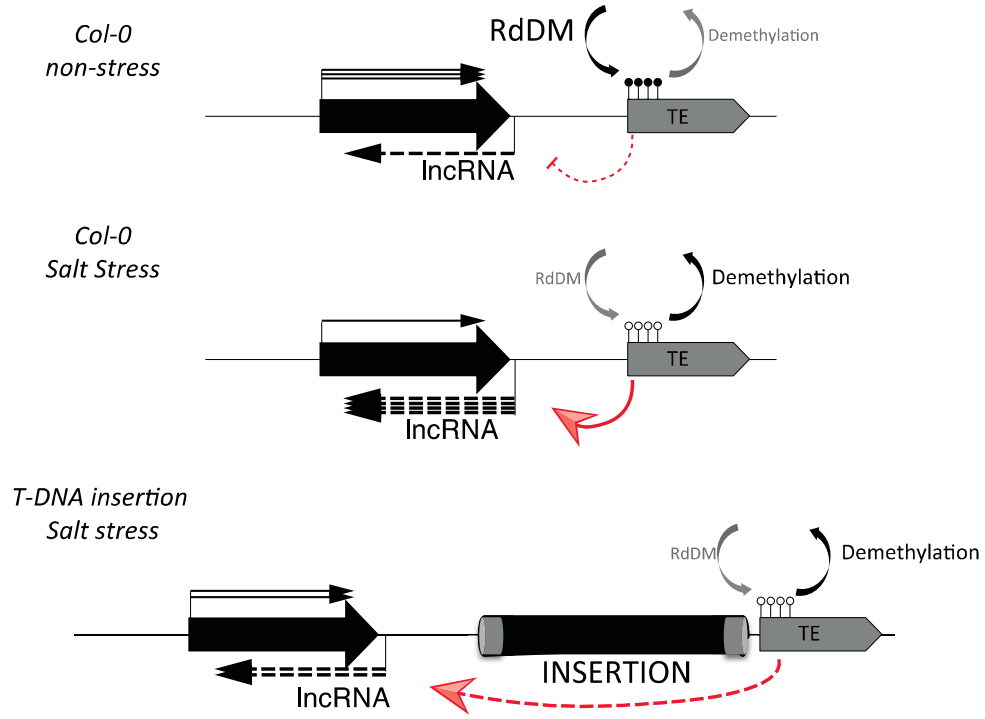


**Figure 25. Proposed model for epigenetic control of CNI1 under stressed and non-stress conditions.** Methylation changes in response to stress are targeted to TE sequence at nearby genes. The RdDM and DNA demethylation pathway regulate the methylation and activity of this TE. Epigenetically controlled TEs might interact with lncRNAs to regulate nearby gene expression under stress. Coding transcripts (black solid arrowed lines), antisense (black dashed arrowed lines), hypermethylated cytosines (black lollipops), hypomethylated cytosines (white lollipops), transgene insertions (grey and black barrel)

## 6.6 Concluding Remarks

The extent to which the environment could contribute to transgenerational epigenetic inheritance and adaptive responses in plants is a debatable topic and a central question in genetics and evolution. Various studies in Arabidopsis suggest that environmental stimuli could direct global changes in DNA methylation and providing plant with novel adaptive benefits (Bilichak et al., 2012; Dowen et al., 2012; Jiang et al., 2014). However, these studies have not been able to fully explain the extent of the proposed epigenetic changes, their mode of inheritance, or their adaptive value to the progeny. Moreover, results from various studies in epigenetic inheritance are conflictive to each other and inconclusive (summarize in Table 3).

**Table 3**. Summary of studies in stress-induced epigenetic inheritance

| **Results from existing studies** | **Source** |
|---|---|
| Several studies have proposed that stress triggers global DNA methylation and that stress-induced epigenetic changes can be stably inherited by the non-stressed. | Molinier et al., 2006; Bilichak et al., 2012; Luna et al., 2012; Rasmann et al., 2012; Slaughter et al., 2012; Jiang et al., 2014. |
| Other studies have reported that stress does not induce heritable changes in DNA methylation and that the primary effects of stress to plant's methylation are transient. | Lang-Mladek et al., 2010; Pecinka et al., 2010; Pecinka and Mittelsten Scheid, 2012; Sani et al., 2013. |
| A few studies have reported genome-wide DNA methylation analyses following stress treatment and showed that stress could induce genome wide methylation changes in plants. However these methylome studies were not combined with transgenerational design and transcriptional or phenotypic analyses, thus the significance of the proposed changes remains unclear. | Dowen et al., 2012; Jiang et al., 2014. |

| | |
|---|---|
| The above conflicting and inconclusive conclusions are being repeatedly questioned as some of the studies contain deficiencies in experimental design and analysis, such as:<br><br>· Stress was applied to plants over their entire lifetime, which could directly impact the developing embryos (offspring) in these plants.<br><br>· Progeny where not grown in the absence of stress over generations, thus the stability and heritability of the epigenetic changes could not be assessed.<br><br>· Statistical analysis of methylation data was not robust enough (the analysis was performed in single plants) thus it is impossible to distinguish between individual-stochastic changes and concrete changes that shared between individual. | Paszkowski and Grossniklaus, 2011; Pecinka and Mittelsten Scheid, 2012; Heard and Martienssen, 2014; Kinoshita and Seki, 2014. |

To elucidate the role of stress in transgenerational adaptation a much more robust and systematic approach is needed, which applies to both the experimental design (ensuring that that developing embryos are not directly exposed to stress, following multiple plants over several generations, including generations without stress exposure) and the analyses (integration of methylome data with phenotypic, transcriptomic and mutations studies). In this study, I performed a systematic DNA methylation analysis of plant populations exposed to salt stress for five consecutive generations followed by non-stress exposure for a further two generations. In addition, I have combined this methylome analysis with phenotypic analyses and molecular studies of the affected loci.

My work has revealed that plants possessed highly dynamic short-term stress memory in the form of DNA methylation changes that affect the expression of stress responsive genes and confers phenotypic-plasticity to the immediate progeny. I have found that repeated exposure to salt stress in the parental lineage could lead to increased tolerance to salt stress in the direct progeny. Some studies proposed that stress-induced epigenetic changes and adaptive

response can be stably inherited across generations and I hypothesize that multigenerational salt stress treatments could lead to heritable methylation changes and adaptive traits. In contrary to my hypothesis and some studies, I found that adaptive response to salt only lasted for one generation and the subsequent non-stressed generation did not shows any improved tolerance to stress. Nevertheless, in accordance to previous studies by Boyko et al (2010) and Luna et al (2012), I have shown that this adaptive response is abolished in mutants defective in the non-CG methylation and DNA demethylation pathways, suggesting that the intergenerational response to salt stress is regulated epigenetically. Whole-genome bisulfite sequencing revealed that salt stress alters CHG and CHH methylation, mostly in form of hypermethylation. Consistent with the observed phenotypes, these methylation changes are present in the immediate progeny but are gradually erased once the stress is alleviated.

I hypothesize that salt-induced methylation marks and adaptive traits will be inherited maternally due to the active resetting of methylation marks in the male gametes. In this study I found that indeed the adaptive responses to salt stress are primarily inherited through maternal transmission. Whole-genome bisulfite sequencing on the male gametes revealed that salt-induced DMRs are being reset in the male germline by the activity of DNA glycosylases (DME). Previous work published by Calarco et al. (2012) had shown that DME is involved in resetting of DNA methylation in the male germline, however the implications of such resetting were not clear. My data reveal a significant biological role for this mechanism: the resetting of epigenetic changes induced by stress. This finding highlight the significant differences in the way males and females transfer newly acquired epigenetic changes to offspring in plants, and this may extend to other sexually reproducing organisms.

Finally, I hypothesize that stress-induced epigenetic changes are not occurred stochastically but targeted to certain stress-responsive genic regions or TEs adjacent to genes. These targeted methylation changes might alter the transcriptional response of stress related genes and providing plants with novel adaptive/phenotypic response to stress. Interestingly, half of

salt-induced DMRs identified in this study are overlapped with spontaneous DMRs present in plants grown under controlled environment conditions, thus suggesting that certain regions of the genome are epigenetically labile and prone to methylation changes. I found that salt-induced DMRs are primarily targeted to TEs sequences located nearby protein-coding genes. I have provided evidence that salt-induced DMRs affect the transcriptional responsiveness of salt-regulated genes and that an integral component of this regulation is mediated by the activity of antisense lncRNAs.

In summary, this study clarified how plants are able to respond and adapt to stress and explain the regulatory mechanisms by which these stress response occurs. As proposed in the aims, I have addressed three important questions in the field of epigenetic inheritance: the extent of stress-induced epigenetic changes, their mode of inheritance and their adaptive value to the progeny. The robust conclusions drawn from this study hopefully would provide the necessary insight to understand stress memory mechanism in plants and have immense implications for future studies in plant and animal assisted breeding and reproduction.

# 7. Appendix

## 7.1 Appendix for Chapter 1

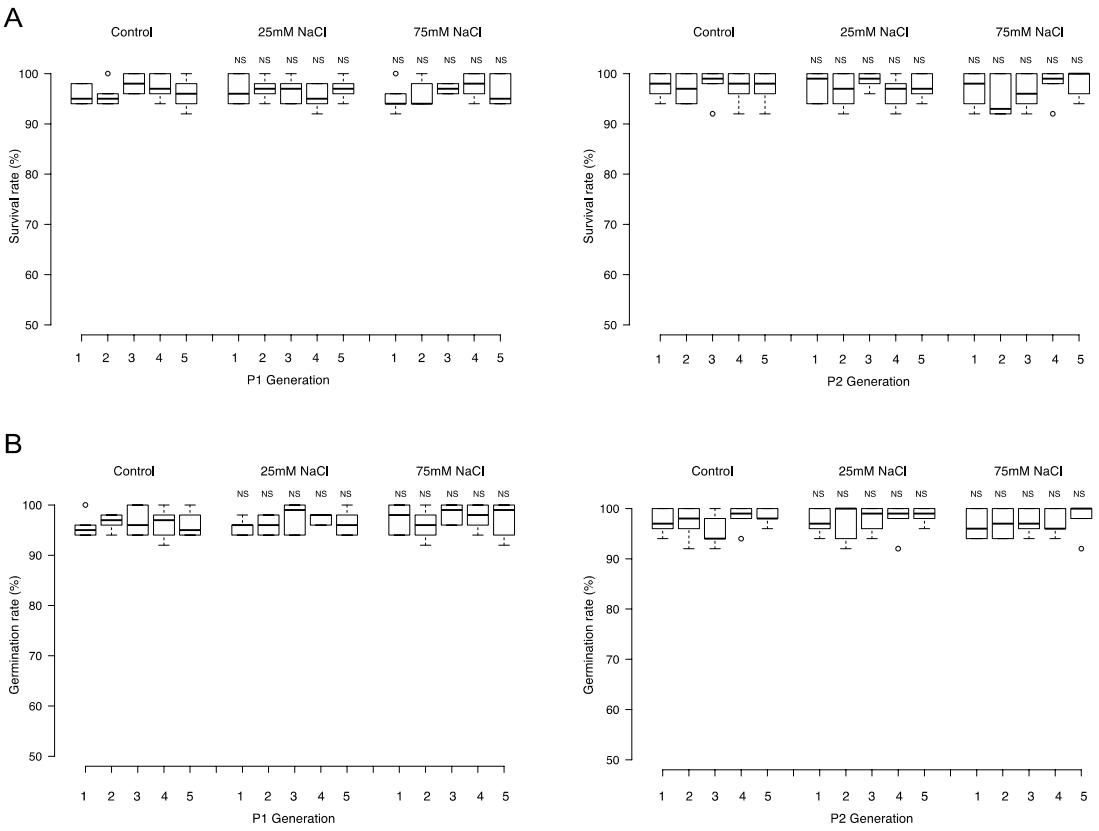


**Figure 26. Survival and germination rate on MS media without salt for P1 and P2 progeny of control and salt-treated plants** (A) Survival rates of P1 and P2 plants on medium without salt. For each sample and treatment I analysed 6 plates and assessed 50 seedlings per plate. B) Germination rates of P1 and P2 seeds on medium without salt. For each sample and treatment I analysed 6 plates and assessed 50 seed per plate. (unpaired Student's *t*-test; ** $p<0.01$; * $p<0.05$; NS $p>0.05$).

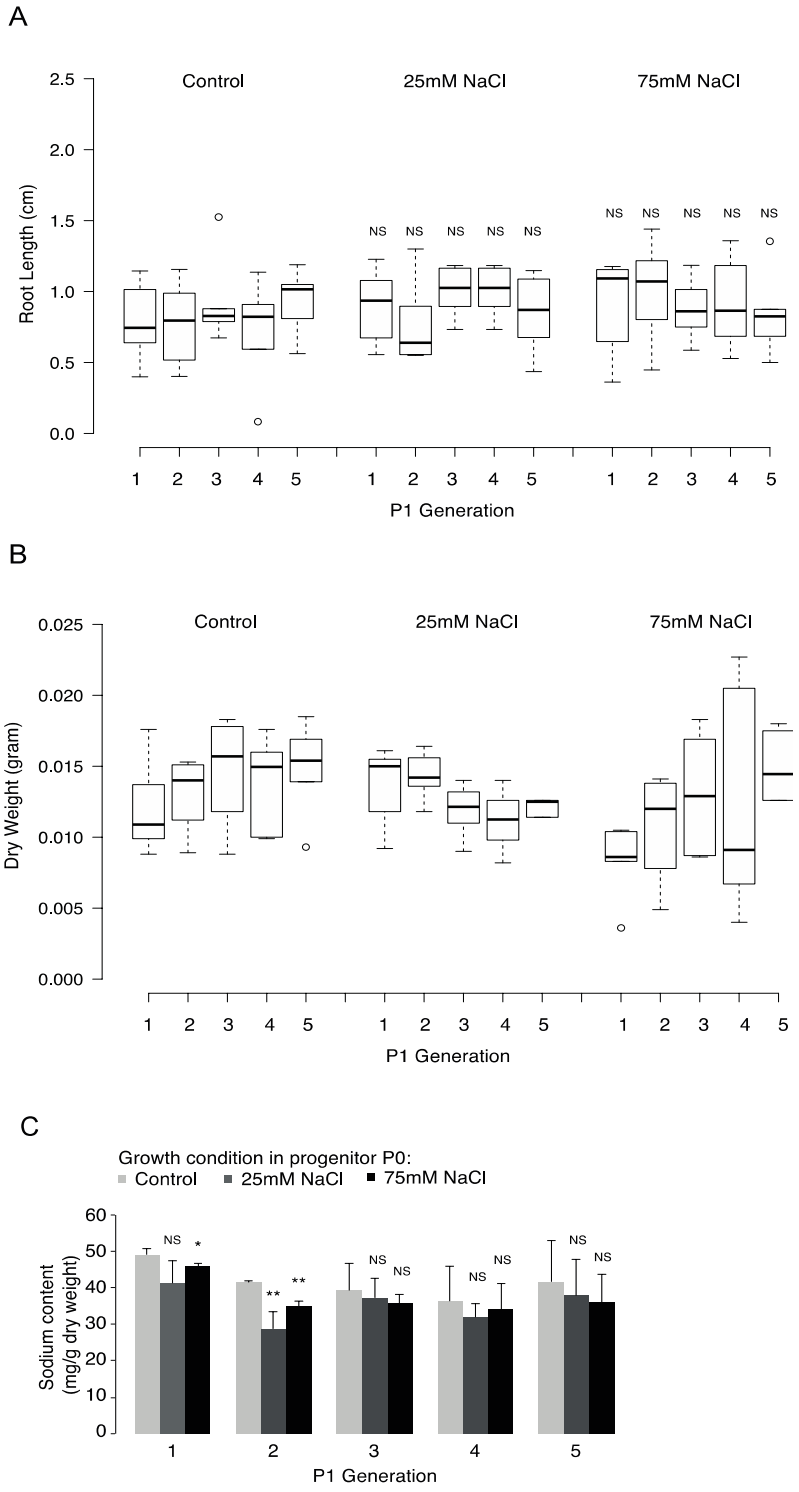


**Figure 27. High-salinity tolerance assays** (A) Root elongation of P1 plants grown on medium supplemented with 175 mM NaCl for two weeks. For each sample and treatment I analysed 10 plants. (B) Aerial dry weight of P1 plants grown for 5 weeks on medium supplemented with 100 mM NaCl. For each sample and treatment I analysed 10 plants (C) Sodium content of P1 plants grown for 5 weeks medium supplemented with 100 mM NaCl. For each sample and treatment I analysed 10 plants. Asterisks indicate a significant difference to the control of the same generation (unpaired Student's *t*-test; ** $p<0.01$; * $p<0.05$; NS $p>0.05$)

## 7.2 Appendix for Chapter 2

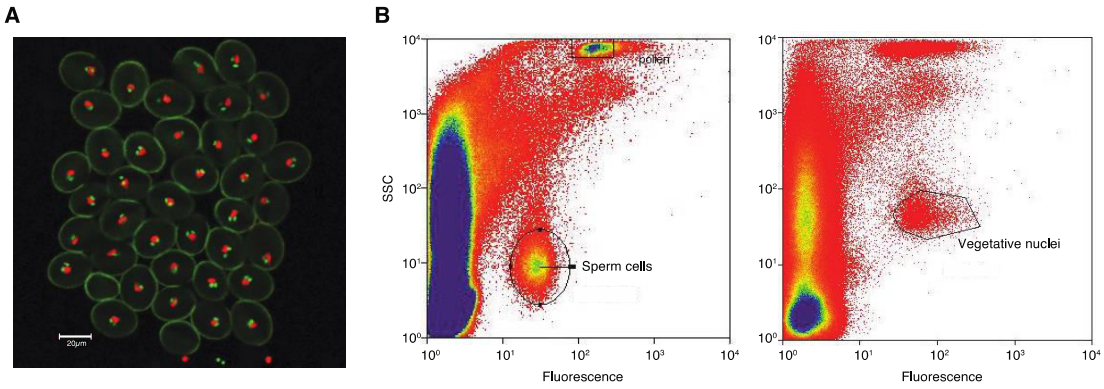


**Figure 28. Isolation of sperm cells and vegetative cells by fluorescent-activated-cell sorting.** (A) Confocal microscope image (25x) of pollen from the *A. thaliana pMGH3::MGH3-eGFP/pACT11::H2B* marker line. *pMGH3::MGH3-eGFP* expression marks the sperm cell nuclei (green); *pACT11p::H2B-mRFP* expression labels vegetative cell nuclei (red). (B) Isolation of sperm and vegetative cells by Fluorescence-Activated-Cell-Sorting (FACS). Sperm cells and vegetative nuclei were isolated based on their GFP and RFP signal, respectively, as well as on their intra-cellular complexity (side scatter, SSC) andparticle size (forward scatter, FSC).

**Table 4.** Sequences of used primers for qPCR.

| Gene | Name | Forward | Reverse | Comment |
|---|---|---|---|---|
| At1g13320 | PROTEIN PHOSPHATASE 2A SUBUNIT A3 | TAACGTGGCCAAAATGATGC | GTTCTCCACAACCGCTTGGT | Housekeeping |
| At1g13440 | GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C-2 | TTGGTGACAACAGGTCCAAGCA | AAACTTGTCGCTCAATGCAATC | Housekeeping |
| At3g62250 | UBIQUITIN 5 | CTCCTTCTTTCTGGTAAACGT | GGTGCTAAGAAGAGGAAGAAT | Housekeeping |
| At1g66230 | MYB DOMAIN PROTEIN 20 | CTTCGCATTCTTCTAATGATTCGAG | GTTTCCCCCACCCCATAGTTTC | Gene of interest |
| At2g33380.1 | CALEOSIN 3 | AATGGCAATCGATCCTTTTG | AGAATTGGCCCTCTCTTTGG | Gene of interest |
| At2g33380.2 | CALEOSIN 3 (splice variant) | CCGAAGGAAGGCTTTCAAAC | TTCGCTAACCAAACACACACA | Gene of interest |
| At5g27420 | CARBON/NITROGEN INSENSITIVE 1 | ACCGGTGGGCTTTTCTTAG | GGAACCGCTAGTTGAACCAA | Gene of interest |

**Table 5.** Methylation sequencing statistic

| Type od Samples | Sample Name | Unique Mapped Read | Total position covered | Methylated position | False methylation rate (%) | Average coverage per strand |
|---|---|---|---|---|---|---|
| **Leaf Generation 1** | P0 control | 28,983,681 | 38,670,050 | 3,182,011 | 0.1 | 7.0 |
| | P0 control | 35,841,097 | 40,340,754 | 3,907,426 | 0.2 | 9.2 |
| | P0 25mM NaCl | 56,458,202 | 41,347,647 | 5,063,170 | 0.2 | 14.1 |
| | P0 25mM NaCl | 52,293,803 | 41,309,997 | 4,884,613 | 0.1 | 13.1 |
| | P0 75mM NaCl | 53,842,470 | 41,382,468 | 4,718,789 | 0.2 | 13.7 |
| | P0 75mM NaCl | 29,824,742 | 39,310,462 | 3,474,981 | 0.1 | 7.6 |
| | P1 control | 57,070,675 | 40,874,831 | 3,763,129 | 0.2 | 10.9 |
| | P1 control | 42,032,174 | 40,930,293 | 3,905,609 | 0.2 | 10.3 |
| | P1 25mM NaCl | 76,676,411 | 41,345,382 | 4,079,292 | 0.1 | 14.5 |
| | P1 25mM NaCl | 73,943,062 | 41,395,876 | 4,099,685 | 0.2 | 14.5 |
| | P1 75mM NaCl | 45,991,898 | 40,887,801 | 3,838,083 | 0.2 | 10.3 |
| | P1 75mM NaCl | 66,936,364 | 41,339,875 | 4,029,501 | 0.2 | 13.4 |
| | P2 control | 51,805,075 | 41,200,543 | 4,002,022 | 0.1 | 12.2 |
| | P2 control | 69,215,535 | 41,443,176 | 4,362,206 | 0.1 | 15.6 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | P2 25mM NaCl | 47,177,182 | 41,296,589 | 4,258,970 | 0.1 | 12.6 |
| | P2 25mM NaCl | 54,257,209 | 41,383,875 | 4,351,300 | 0.1 | 13.2 |
| | P2 75mM NaCl | 44,154,313 | 41,130,082 | 4,141,155 | 0.1 | 11.5 |
| | P2 75mM NaCl | 39,345,129 | 40,866,870 | 4,016,009 | 0.1 | 10.5 |
| **Leaf Generation 3** | P0 control | 62,751,764 | 41,464,833 | 4,413,323 | 0.1 | 14.7 |
| | P0 control | 52,487,985 | 41,148,263 | 4,349,973 | 0.1 | 12.0 |
| | P0 25mM NaCl | 33,713,766 | 40,232,216 | 3,935,499 | 0.2 | 9.0 |
| | P0 25mM NaCl | 84,000,952 | 41,572,656 | 4,559,247 | 0.2 | 20.2 |
| | P0 75mM NaCl | 63,840,088 | 41,466,409 | 4,514,102 | 0.1 | 15.3 |
| | P0 75mM NaCl | 75,609,547 | 41,510,632 | 4,610,325 | 0.1 | 16.9 |
| | P1 control | 47,218,625 | 41,272,040 | 4,336,901 | 0.2 | 12.8 |
| | P1 control | 111,203,920 | 41,616,065 | 4,592,941 | 0.1 | 25.6 |
| | P1 25mM NaCl | 75,082,045 | 41,507,740 | 4,315,310 | 0.2 | 16.4 |
| | P1 25mM NaCl | 85,717,462 | 41,536,185 | 4,423,679 | 0.4 | 18.3 |
| | P1 75mM NaCl | 64,728,765 | 41,299,786 | 4,334,413 | 0.3 | 14.4 |
| | P1 75mM NaCl | 70,618,570 | 41,515,154 | 4,529,256 | 0.2 | 15.8 |
| | P2 control | 48,001,452 | 41,159,571 | 4,287,742 | 0.2 | 13.0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | P2 control | 55,651,008 | 41,429,732 | 4,325,844 | 0.2 | 14.4 |
| | P2 25mM NaCl | 56,841,052 | 41,456,062 | 4,457,029 | 0.1 | 14.6 |
| | P2 25mM NaCl | 67,412,861 | 41,569,539 | 4,557,617 | 0.2 | 17.9 |
| | P2 75mM NaCl | 53,095,056 | 41,406,861 | 4,405,853 | 0.1 | 14.0 |
| | P2 75mM NaCl | 59,973,046 | 41,452,821 | 4,545,497 | 0.2 | 14.3 |
| **Leaf Generation 5** | P0 control | 36,839,456 | 41,165,550 | 4,028,275 | 0.2 | 11.7 |
| | P0 control | 37,041,378 | 40,824,604 | 3,815,796 | 0.1 | 10.5 |
| | P0 25mM NaCl | 45,709,486 | 41,388,855 | 4,683,843 | 0.2 | 14.7 |
| | P0 25mM NaCl | 49,666,057 | 40,754,767 | 4,316,538 | 0.1 | 10.5 |
| | P0 75mM NaCl | 47,027,759 | 39,637,246 | 3,916,505 | 0.1 | 7.9 |
| | P0 75mM NaCl | 43,796,684 | 41,135,466 | 4,575,475 | 0.2 | 11.6 |
| | P1 control | 46,230,055 | 40,920,145 | 4,107,999 | 0.1 | 10.4 |
| | P1 control | 39,600,977 | 41,293,982 | 4,490,083 | 0.2 | 13.3 |
| | P1 25mM NaCl | 97,328,377 | 40,201,891 | 3,902,532 | 0.1 | 8.8 |
| | P1 25mM NaCl | 29,165,443 | 41,072,556 | 4,130,515 | 0.2 | 11.8 |
| | P1 75mM NaCl | 44,547,212 | 40,953,599 | 4,144,900 | 0.1 | 11.0 |
| | P1 75mM NaCl | 80,146,775 | 40,908,856 | 4,095,363 | 0.1 | 10.8 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | P2 control | 66,325,211 | 40,563,765 | 3,852,310 | 0.4 | 9.4 |
| | P2 control | 59,568,523 | 39,629,756 | 3,316,159 | 0.2 | 7.9 |
| | P2 25mM NaCl | 48,072,587 | 40,108,754 | 3,550,640 | 0.2 | 8.6 |
| | P2 25mM NaCl | 59,817,442 | 40,798,675 | 3,948,590 | 0.3 | 10.4 |
| | P2 75mM NaCl | 113,846,071 | 39,382,576 | 3,384,588 | 0.3 | 7.6 |
| | P2 75mM NaCl | 54,909,924 | 40,809,009 | 4,012,327 | 0.4 | 10.4 |
| **Pollen** | Sperm cell, control | 36,839,456 | 40,835,684 | 3,779,006 | 0.2 | 9.9 |
| | Vegetative nucleus, control | 37,041,378 | 40,104,367 | 3,521,209 | 0.2 | 8.4 |
| | Sperm cell, control | 45,709,486 | 41,365,556 | 4,097,202 | 0.2 | 12.7 |
| | Vegetative nucleus, control | 49,666,057 | 41,291,117 | 5,190,437 | 0.0 | 12.5 |
| | Sperm cell, control | 47,027,759 | 41,383,786 | 4,073,397 | 0.4 | 13.1 |
| | Vegetative nucleus, control | 43,796,684 | 40,946,368 | 4,783,877 | 0.1 | 10.5 |
| | Sperm cell, 25mM NaCl | 46,230,055 | 40,836,851 | 3,628,454 | 0.1 | 9.4 |
| | Vegetative nucleus 25mM NaCl | 39,600,977 | 40,991,237 | 4,833,453 | 0.2 | 10.7 |
| | Sperm cell, 25mM NaCl | 97,328,377 | 41,566,516 | 4,078,123 | 0.5 | 19.8 |
| | Vegetative nucleus 25mM NaCl | 29,165,443 | 40,192,000 | 4,454,728 | 0.1 | 8.3 |
| | Sperm cell, 25mM NaCl | 44,547,212 | 41,358,961 | 4,071,061 | 0.1 | 12.8 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Vegetative nucleus 25mM NaCl | 80,146,775 | 35,223,306 | 2,560,066 | 0.5 | 5.2 |
| | Sperm cell, 75mM NaCl | 66,325,211 | 41,522,954 | 4,205,932 | 0.2 | 17.9 |
| | Vegetative nucleus 75mM NaCl | 59,568,523 | 40,877,136 | 4,591,614 | 0.1 | 10.4 |
| | Sperm cell, 75mM NaCl | 48,072,587 | 41,362,886 | 4,107,841 | 0.2 | 13.4 |
| | Vegetative nucleus 75mM NaCl | 59,817,442 | 40,688,978 | 4,422,896 | 0.4 | 9.8 |
| | Sperm cell, 75mM NaCl | 113,846,071 | 41,533,632 | 3,992,754 | 0.2 | 16.5 |
| | Vegetative nucleus 75mM NaCl | 54,909,924 | 28,868,659 | 1,784,740 | 0.2 | 3.8 |

## 7.3 Appendix for Chapter 4

**Table 6.** Differentially Methylated Regions

| Type of DMRs | Chromosome | Start position | Region length (bp) | p-value for DMR | Methylation contexts |
|---|---|---|---|---|---|
| **DMRs Generation 1** | 1 | 6425924 | 637 | 1.31E-05 | CHG |
| | 1 | 12525123 | 202 | 2.41E-16 | CG,CHG |
| | 1 | 14152895 | 93 | 5.38E-05 | CG |
| | 1 | 17376946 | 290 | 9.00E-05 | CHG |
| | 1 | 26771320 | 180 | 3.20E-05 | CG,CHH |
| | 1 | 29250129 | 176 | 1.45E-08 | CHG |
| | 2 | 303242 | 60 | 6.45E-05 | CHG,CHH |
| | 2 | 722082 | 321 | 0.000138663 | CHG |
| | 2 | 1504629 | 210 | 8.97E-06 | CHH |
| | 2 | 3270315 | 328 | 0.00040288 | CHG |
| | 2 | 3312699 | 369 | 7.81E-12 | CG |
| | 2 | 3316723 | 285 | 0.000170826 | CG,CHG |
| | 2 | 6709181 | 93 | 3.79E-08 | CG,CHG |
| | 2 | 7278817 | 106 | 6.45E-05 | CHG |

| | | | | | |
|---|---|---|---|---|---|
| | 2 | 8366454 | 301 | 3.35E-05 | CHH |
| | 2 | 9333493 | 346 | 0.000415341 | CG,CHG |
| | 2 | 10932978 | 78 | 0.000415341 | CG,CHG,CHH |
| | 2 | 12833632 | 206 | 6.31E-06 | CG,CHG |
| | 2 | 15140190 | 210 | 0.000386213 | CG,CHG |
| | 2 | 17530747 | 271 | 1.08E-06 | CG |
| | 3 | 7355346 | 526 | 0.000415341 | CG |
| | 3 | 7703993 | 256 | 1.83E-07 | CG,CHG |
| | 3 | 8379766 | 245 | 0.000212753 | CHG |
| | 3 | 9149001 | 92 | 0.00040288 | CHG,CHH |
| | 3 | 10062730 | 120 | 0.00038971 | CG |
| | 3 | 12235279 | 796 | 1.50E-05 | CG |
| | 3 | 12917666 | 923 | 0.000154744 | CG |
| | 3 | 15912134 | 237 | 5.38E-05 | CG |
| | 3 | 16526447 | 301 | 9.09E-05 | CG |
| | 3 | 16911034 | 233 | 0.000206392 | CHG |
| | 4 | 1507861 | 179 | 0.000426008 | CHG |

| | | | | | |
|---|---|---|---|---|---|
| | 4 | 2626032 | 242 | 4.34E-09 | CG |
| | 4 | 5987052 | 277 | 6.61E-06 | CHG,CHH |
| | 4 | 6287481 | 94 | 0.000427845 | CG,CHH |
| | 4 | 10649070 | 193 | 2.71E-06 | CG |
| | 4 | 13681401 | 216 | 0.000513412 | CHG |
| | 4 | 16320873 | 211 | 1.10E-12 | CG,CHG,CHH |
| | 5 | 3202808 | 75 | 3.61E-05 | CG |
| | 5 | 6398917 | 100 | 2.04E-07 | CG,CHG |
| | 5 | 9220194 | 385 | 4.11E-10 | CG,CHG |
| | 5 | 15075157 | 166 | 4.54E-09 | CG |
| | 5 | 15196575 | 283 | 1.15E-05 | CHG |
| | 5 | 17107616 | 140 | 9.09E-05 | CG,CHH |
| | 5 | 19001367 | 660 | 4.56E-08 | CG |
| | 5 | 19002123 | 229 | 2.96E-05 | CG |
| | 5 | 20266876 | 89 | 0.000134439 | CHG,CHH |
| | 5 | 22069237 | 155 | 0.000168177 | CHG |
| | 5 | 22255885 | 108 | 0.000212753 | CHG |

| | | | | | |
|---|---|---|---|---|---|
| | 5 | 23340073 | 125 | 0.000170826 | CG |
| **DMRs Generation 3** | 1 | 1592439 | 175 | 5.90E-12 | CHG |
| | 1 | 2993786 | 78 | 4.81E-05 | CHG |
| | 1 | 3785252 | 478 | 0.00021835 | CG |
| | 1 | 3919896 | 306 | 0.000313548 | CHG |
| | 1 | 4093182 | 362 | 0.000147283 | CG |
| | 1 | 4293160 | 66 | 4.36E-08 | CG,CHG |
| | 1 | 5359011 | 164 | 1.51E-06 | CHG |
| | 1 | 7313036 | 207 | 9.80E-06 | CHG |
| | 1 | 8452237 | 446 | 6.92E-17 | CHG |
| | 1 | 9081652 | 94 | 1.70E-15 | CHH |
| | 1 | 9173714 | 126 | 0.000383273 | CG,CHG |
| | 1 | 11422010 | 315 | 1.29E-05 | CG |
| | 1 | 12055247 | 55 | 0.000266391 | CHG |
| | 1 | 13282141 | 204 | 8.90E-05 | CHG |
| | 1 | 14660183 | 1070 | 7.32E-05 | CG |
| | 1 | 14737413 | 2851 | 7.90E-05 | CG |

| | | | | | |
|---|---|---|---|---|---|
| | 1 | 16779452 | 139 | 4.22E-08 | CHH |
| | 1 | 17091714 | 116 | 1.16E-13 | CHG,CHH |
| | 1 | 19352703 | 90 | 4.10E-05 | CG |
| | 1 | 19362933 | 200 | 0.000266391 | CHG |
| | 1 | 20106655 | 212 | 1.16E-19 | CG |
| | 1 | 20462586 | 223 | 3.49E-14 | CHG |
| | 1 | 20468078 | 246 | 2.45E-14 | CG |
| | 1 | 20800468 | 106 | 1.30E-14 | CHG |
| | 1 | 21933036 | 83 | 2.31E-12 | CHH |
| | 1 | 22075267 | 101 | 6.83E-10 | CG,CHG |
| | 1 | 22356980 | 69 | 1.41E-11 | CHG,CHH |
| | 1 | 22592602 | 231 | 0.000344259 | CG |
| | 1 | 23004561 | 67 | 5.02E-06 | CHG |
| | 1 | 23519500 | 129 | 6.30E-23 | CG,CHG |
| | 1 | 23946942 | 163 | 2.94E-06 | CG |
| | 1 | 24675742 | 137 | 2.73E-25 | CHG,CHH |
| | 1 | 25217372 | 101 | 1.56E-09 | CHG |

| | | | | | |
|---|---|---|---|---|---|
| | 1 | 27556180 | 136 | 6.23E-28 | CG,CHG,CHH |
| | 1 | 27556318 | 145 | 1.04E-10 | CHG |
| | 1 | 28788962 | 655 | 0.000356663 | CHG |
| | 1 | 28843236 | 99 | 5.54E-11 | CG,CHG,CHH |
| | 1 | 29250118 | 168 | 3.41E-05 | CG |
| | 2 | 1361014 | 287 | 7.75E-05 | CHG |
| | 2 | 4889809 | 289 | 0.000304732 | CHG |
| | 2 | 5611774 | 344 | 2.50E-09 | CHG |
| | 2 | 6709181 | 93 | 3.35E-07 | CG |
| | 2 | 7060764 | 162 | 1.94E-12 | CG |
| | 2 | 7299011 | 178 | 0.000247886 | CHG |
| | 2 | 7875160 | 275 | 8.77E-08 | CHG |
| | 2 | 7963665 | 154 | 0.000184435 | CHG |
| | 2 | 10558196 | 345 | 1.41E-07 | CHG |
| | 2 | 11246500 | 79 | 0.000158784 | CHH |
| | 2 | 11669090 | 74 | 6.44E-05 | CHH |
| | 2 | 11762585 | 95 | 4.00E-05 | CG |

| | | | | | |
|---|---|---|---|---|---|
| | 2 | 11822627 | 116 | 5.18E-18 | CG,CHG |
| | 2 | 12202403 | 153 | 2.70E-09 | CHG |
| | 2 | 12773633 | 633 | 4.38E-11 | CG |
| | 2 | 13310472 | 174 | 4.52E-06 | CHG |
| | 2 | 13675779 | 165 | 1.57E-05 | CG,CHG |
| | 2 | 14145283 | 126 | 1.08E-05 | CG |
| | 2 | 14386655 | 511 | 6.54E-22 | CG |
| | 2 | 14392836 | 296 | 0.000266391 | CG |
| | 2 | 15094270 | 129 | 3.57E-08 | CHG |
| | 2 | 16082688 | 128 | 2.00E-06 | CHG |
| | 2 | 16143198 | 139 | 1.92E-12 | CHG |
| | 2 | 16253343 | 135 | 8.07E-05 | CHG |
| | 2 | 18803926 | 144 | 1.36E-15 | CG |
| | 3 | 810431 | 75 | 0.000329623 | CHH |
| | 3 | 1122051 | 94 | 0.000181597 | CHG |
| | 3 | 2211885 | 123 | 2.65E-10 | CG,CHG |
| | 3 | 3410240 | 83 | 2.07E-11 | CHH |

| | | | | | |
|---|---|---|---|---|---|
| | 3 | 4729457 | 284 | 5.73E-07 | CG |
| | 3 | 5983234 | 118 | 2.20E-11 | CHG |
| | 3 | 5984842 | 93 | 6.44E-13 | CHG,CHH |
| | 3 | 6246907 | 204 | 4.68E-21 | CHG |
| | 3 | 6752142 | 98 | 0.00046664 | CHG |
| | 3 | 7785867 | 578 | 0.000182847 | CG,CHG |
| | 3 | 9130664 | 109 | 1.52E-06 | CHH |
| | 3 | 9149001 | 77 | 0.000150769 | CHG |
| | 3 | 9334843 | 120 | 8.28E-12 | CG,CHG |
| | 3 | 9874307 | 150 | 1.36E-15 | CHG |
| | 3 | 11316805 | 684 | 0.00017936 | CG |
| | 3 | 12062478 | 124 | 0.000247886 | CHH |
| | 3 | 12559226 | 88 | 5.15E-05 | CHG |
| | 3 | 13451631 | 645 | 0.000151749 | CG |
| | 3 | 17546375 | 288 | 0.000257678 | CHG |
| | 3 | 18310526 | 341 | 1.36E-07 | CHG |
| | 3 | 18791056 | 130 | 1.60E-30 | CHG |

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|---|---|---|---|---|---|
| | 3 | 21457352 | 61 | 6.02E-08 | CHG,CHH |
| | 3 | 21664707 | 101 | 1.92E-09 | CHG |
| | 4 | 44016 | 121 | 3.06E-16 | CG,CHG,CHH |
| | 4 | 712862 | 108 | 8.77E-08 | CHG |
| | 4 | 2036351 | 196 | 1.41E-11 | CHG,CHH |
| | 4 | 2181856 | 166 | 1.20E-07 | CG,CHG |
| | 4 | 5987060 | 260 | 2.69E-14 | CG,CHH |
| | 4 | 6312657 | 257 | 0.000372782 | CHG |
| | 4 | 6808875 | 176 | 0.000330672 | CHG |
| | 4 | 7843785 | 49 | 0.000432013 | CHG,CHH |
| | 4 | 7844351 | 463 | 2.91E-06 | CG |
| | 4 | 8189130 | 32 | 0.0003677 | CHG |
| | 4 | 8208257 | 125 | 0.000115812 | CHG |
| | 4 | 8271308 | 153 | 1.04E-26 | CG |
| | 4 | 8836493 | 679 | 3.18E-07 | CG |
| | 4 | 9791570 | 123 | 7.69E-06 | CG |
| | 4 | 10648858 | 198 | 0.000356028 | CHG |

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|---|---|---|---|---|---|
| | 4 | 11255674 | 181 | 8.73E-06 | CG |
| | 4 | 12302980 | 178 | 8.10E-05 | CHG |
| | 4 | 14432737 | 33 | 1.81E-07 | CHH |
| | 4 | 14432772 | 119 | 1.54E-15 | CHG |
| | 4 | 16050135 | 53 | 0.000114912 | CHH |
| | 5 | 911259 | 44 | 0.000133918 | CHG |
| | 5 | 911442 | 56 | 4.52E-06 | CHH |
| | 5 | 951554 | 115 | 1.38E-07 | CG,CHG |
| | 5 | 2252976 | 61 | 4.54E-07 | CHH |
| | 5 | 6109988 | 120 | 0.000266391 | CHG |
| | 5 | 7027425 | 158 | 2.61E-11 | CG |
| | 5 | 7655121 | 164 | 3.40E-10 | CHG |
| | 5 | 7806665 | 216 | 6.92E-17 | CG,CHG |
| | 5 | 8362410 | 105 | 1.04E-09 | CHG |
| | 5 | 9042742 | 104 | 7.84E-05 | CHH |
| | 5 | 9049762 | 174 | 6.47E-19 | CG,CHG,CHH |
| | 5 | 9115201 | 112 | 0.000181586 | CHG |

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|---|---|---|---|---|---|
| | 5 | 9280468 | 185 | 3.08E-06 | CG |
| | 5 | 9551008 | 145 | 3.23E-13 | CG |
| | 5 | 9646196 | 113 | 5.38E-05 | CG |
| | 5 | 9686262 | 157 | 2.30E-08 | CG |
| | 5 | 9883556 | 90 | 0.000248198 | CHH |
| | 5 | 10609542 | 265 | 3.77E-09 | CG |
| | 5 | 10654019 | 209 | 6.02E-08 | CHG,CHH |
| | 5 | 15120611 | 30 | 8.43E-05 | CHH |
| | 5 | 15145713 | 464 | 0.000279233 | CHG |
| | 5 | 15755571 | 228 | 4.44E-08 | CG,CHG |
| | 5 | 15796047 | 240 | 2.49E-11 | CHG |
| | 5 | 15800478 | 113 | 1.65E-06 | CHG |
| | 5 | 16142031 | 116 | 1.53E-10 | CHH |
| | 5 | 16963754 | 111 | 6.80E-13 | CG,CHH |
| | 5 | 17710709 | 163 | 0.000449437 | CHG |
| | 5 | 17916879 | 72 | 6.85E-08 | CHG,CHH |
| | 5 | 18352757 | 108 | 1.07E-07 | CHH |

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|---|---|---|---|---|---|
| | 5 | 18778129 | 126 | 2.48E-09 | CHH |
| | 5 | 20377175 | 108 | 1.58E-06 | CG,CHH |
| | 5 | 20527715 | 170 | 4.00E-23 | CG,CHG,CHH |
| | 5 | 20839706 | 90 | 8.73E-06 | CG,CHG |
| | 5 | 21423637 | 352 | 1.28E-06 | CHG |
| | 5 | 21821003 | 384 | 3.48E-07 | CHG |
| | 5 | 21855333 | 197 | 5.28E-14 | CHH |
| | 5 | 22069217 | 175 | 1.15E-05 | CHG |
| | 5 | 22429971 | 34 | 1.09E-06 | CHH |
| | 5 | 22703532 | 130 | 5.40E-05 | CHG |
| | 5 | 23340005 | 192 | 6.69E-06 | CG |
| | 5 | 24526826 | 191 | 2.35E-08 | CG |
| | 5 | 25469493 | 432 | 8.61E-07 | CG |
| **DMR generation 5** | 1 | 781073 | 136 | 4.12E-09 | CHG |
| | 1 | 2335177 | 106 | 4.49E-05 | CHG |
| | 1 | 3595919 | 110 | 0.000357676 | CHG,CHH |
| | 1 | 6233902 | 1271 | 1.27E-05 | CHG |

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|---|---|---|---|---|---|
| | 1 | 7201794 | 246 | 2.98E-05 | CHG |
| | 1 | 8471923 | 279 | 0.000303737 | CHG |
| | 1 | 9062087 | 536 | 9.55E-05 | CHG |
| | 1 | 9253887 | 296 | 5.79E-18 | CHG |
| | 1 | 9454045 | 105 | 5.38E-07 | CHG |
| | 1 | 10005795 | 215 | 0.000700307 | CHG,CHH |
| | 1 | 11422000 | 411 | 0.000441964 | CG |
| | 1 | 14687577 | 361 | 3.13E-05 | CHG |
| | 1 | 14688123 | 813 | 1.40E-05 | CG |
| | 1 | 14699368 | 1071 | 3.13E-05 | CG |
| | 1 | 14932724 | 798 | 0.000847572 | CG |
| | 1 | 14939796 | 838 | 7.20E-06 | CG |
| | 1 | 14945023 | 720 | 1.06E-05 | CG |
| | 1 | 14953689 | 1005 | 0.000232347 | CG |
| | 1 | 14987545 | 1305 | 0.000656363 | CHG |
| | 1 | 15010023 | 641 | 0.000357676 | CHG |
| | 1 | 16125413 | 162 | 0.000885244 | CG,CHG |

| | | | | | |
|---|---|---|---|---|---|
| | 1 | 17143850 | 203 | 0.000428344 | CHG |
| | 1 | 17386976 | 211 | 2.27E-05 | CHG |
| | 1 | 19226337 | 280 | 1.26E-05 | CG |
| | 1 | 22075104 | 264 | 3.84E-06 | CG,CHG |
| | 1 | 23004550 | 87 | 1.43E-07 | CHG,CHH |
| | 1 | 23087814 | 171 | 1.54E-18 | CG,CHH |
| | 1 | 24041433 | 116 | 4.04E-11 | CHH |
| | 1 | 24506439 | 247 | 0.0002985 | CHG |
| | 1 | 24675721 | 158 | 5.54E-13 | CHG |
| | 1 | 25728764 | 156 | 0.001046 | CHG |
| | 1 | 25879497 | 158 | 3.13E-05 | CHH |
| | 1 | 27059834 | 95 | 0.000148537 | CG,CHG |
| | 1 | 27121072 | 98 | 1.08E-05 | CHH |
| | 1 | 27556171 | 224 | 3.36E-08 | CHG |
| | 1 | 28843953 | 199 | 1.26E-07 | CG |
| | 1 | 29316490 | 154 | 0.000486841 | CHG |
| | 2 | 473786 | 280 | 2.99E-05 | CHG |

| | | | | | |
|---|---|---|---|---|---|
| | 2 | 592888 | 205 | 0.000303737 | CHG |
| | 2 | 2241179 | 29 | 0.000806995 | CHH |
| | 2 | 3194503 | 925 | 3.64E-06 | CHG |
| | 2 | 3647475 | 677 | 0.000806995 | CHG |
| | 2 | 4538137 | 438 | 0.000886256 | CG |
| | 2 | 6397616 | 158 | 2.34E-06 | CG,CHG,CHH |
| | 2 | 7278747 | 151 | 7.56E-07 | CHG |
| | 2 | 8545868 | 364 | 3.94E-05 | CHG |
| | 2 | 9262459 | 125 | 5.01E-05 | CHH |
| | 2 | 9518233 | 186 | 9.15E-08 | CHG |
| | 2 | 9703039 | 123 | 4.11E-05 | CHG |
| | 2 | 11246493 | 67 | 1.15E-05 | CHH |
| | 2 | 11315608 | 289 | 4.65E-06 | CHG |
| | 2 | 11982422 | 33 | 1.32E-05 | CHH |
| | 2 | 12333023 | 129 | 3.89E-07 | CG |
| | 2 | 12409873 | 217 | 0.000930314 | CG |
| | 2 | 14145164 | 254 | 4.09E-10 | CG |

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|---|---|---|---|---|---|
| | 2 | 15140131 | 233 | 8.76E-06 | CG,CHG |
| | 2 | 15332852 | 150 | 6.04E-06 | CHG |
| | 2 | 15511822 | 54 | 7.66E-06 | CG,CHH |
| | 2 | 16588031 | 73 | 1.70E-06 | CHG |
| | 2 | 16742360 | 143 | 7.09E-10 | CHG |
| | 2 | 17530729 | 298 | 8.63E-08 | CHG |
| | 2 | 17788328 | 388 | 0.000303737 | CG |
| | 3 | 756692 | 193 | 2.83E-05 | CHG |
| | 3 | 1712126 | 143 | 2.05E-06 | CHG |
| | 3 | 2258908 | 74 | 4.15E-06 | CHH |
| | 3 | 6172634 | 335 | 0.000463409 | CHG |
| | 3 | 6246892 | 212 | 4.47E-05 | CHG |
| | 3 | 6752149 | 91 | 3.87E-06 | CHG |
| | 3 | 7232564 | 65 | 3.84E-06 | CHG |
| | 3 | 7820206 | 187 | 8.26E-08 | CHG,CHH |
| | 3 | 8032339 | 88 | 0.000304669 | CHG,CHH |
| | 3 | 8903509 | 197 | 0.000374059 | CHG,CHH |

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|---|---|---|---|---|---|
| | 3 | 9334819 | 144 | 5.81E-09 | CHG |
| | 3 | 9473608 | 87 | 4.09E-10 | CHH |
| | 3 | 9906067 | 194 | 0.000675317 | CHG |
| | 3 | 11097214 | 159 | 1.81E-05 | CG |
| | 3 | 11270325 | 226 | 0.000259698 | CG |
| | 3 | 11346267 | 309 | 0.000453304 | CHH |
| | 3 | 13222766 | 197 | 0.000311847 | CHG,CHH |
| | 3 | 13261627 | 115 | 0.000177792 | CHG |
| | 3 | 14319650 | 315 | 0.000935643 | CG |
| | 3 | 15215282 | 225 | 3.38E-07 | CHH |
| | 3 | 15229130 | 513 | 3.39E-06 | CHG |
| | 3 | 16519738 | 285 | 0.000675317 | CHG |
| | 3 | 16887738 | 106 | 0.000160018 | CHH |
| | 3 | 16943038 | 204 | 0.00058767 | CHG |
| | 3 | 18176068 | 122 | 0.000999643 | CHG |
| | 3 | 18884227 | 132 | 1.40E-05 | CHG,CHH |
| | 3 | 20853782 | 158 | 5.81E-09 | CHG,CHH |

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|---|---|---|---|---|---|
| | 3 | 21664709 | 118 | 0.000368997 | CHG |
| | 4 | 44010 | 150 | 3.89E-07 | CG,CHG |
| | 4 | 177170 | 492 | 0.000441964 | CG |
| | 4 | 413221 | 148 | 1.44E-05 | CHG |
| | 4 | 566807 | 113 | 0.000152296 | CHG |
| | 4 | 1303757 | 173 | 1.43E-07 | CHH |
| | 4 | 1339233 | 127 | 0.000900635 | CG |
| | 4 | 1641430 | 272 | 0.000525622 | CHG |
| | 4 | 2036351 | 107 | 2.80E-10 | CG,CHG |
| | 4 | 2423334 | 311 | 0.000148537 | CHH |
| | 4 | 2626084 | 199 | 1.22E-05 | CG |
| | 4 | 4627929 | 445 | 0.000141028 | CHG |
| | 4 | 5298452 | 206 | 0.000806348 | CHG |
| | 4 | 5634255 | 520 | 0.000428344 | CHH |
| | 4 | 5792811 | 135 | 0.000200932 | CHG |
| | 4 | 5938880 | 354 | 0.000374059 | CG |
| | 4 | 6281964 | 242 | 0.001002236 | CHG |

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|---|---|---|---|---|---|
| | 4 | 7243769 | 269 | 0.000806348 | CHG |
| | 4 | 7886816 | 116 | 2.08E-06 | CG,CHG |
| | 4 | 8271310 | 135 | 6.44E-24 | CG,CHG |
| | 4 | 10705502 | 190 | 3.84E-06 | CHG |
| | 4 | 10839785 | 323 | 0.00059712 | CHG |
| | 4 | 11624460 | 123 | 0.00030727 | CHG |
| | 4 | 14432739 | 203 | 2.80E-10 | CHG |
| | 5 | 951575 | 123 | 0.000243281 | CHG |
| | 5 | 1814508 | 144 | 0.000774906 | CHG |
| | 5 | 2353375 | 42 | 0.000806995 | CG,CHH |
| | 5 | 6302200 | 228 | 0.000304669 | CHG |
| | 5 | 7154328 | 325 | 0.000424779 | CG,CHG |
| | 5 | 7478485 | 166 | 4.26E-07 | CHG |
| | 5 | 7777103 | 832 | 1.95E-05 | CG |
| | 5 | 7806735 | 114 | 2.93E-13 | CG,CHG |
| | 5 | 8440235 | 215 | 2.24E-06 | CG,CHG |
| | 5 | 8945223 | 213 | 3.38E-07 | CHH |

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|---|---|---|---|---|---|
| | 5 | 9645559 | 328 | 0.000428344 | CG |
| | 5 | 9686262 | 188 | 0.000416199 | CHH |
| | 5 | 10019776 | 102 | 4.59E-05 | CG,CHH |
| | 5 | 11228950 | 501 | 0.000737194 | CHG |
| | 5 | 11229571 | 513 | 3.41E-08 | CHG |
| | 5 | 11232838 | 1134 | 6.66E-11 | CHG |
| | 5 | 11237990 | 515 | 0.000200932 | CHG |
| | 5 | 11239965 | 375 | 1.27E-05 | CHG |
| | 5 | 11248378 | 486 | 7.43E-06 | CHG |
| | 5 | 11249026 | 401 | 6.77E-06 | CHG |
| | 5 | 11250321 | 391 | 7.85E-05 | CHG |
| | 5 | 11493919 | 412 | 3.06E-05 | CHG |
| | 5 | 12278024 | 573 | 0.000147608 | CHG |
| | 5 | 12325691 | 487 | 0.00075639 | CHG |
| | 5 | 12885975 | 220 | 0.000486841 | CHG |
| | 5 | 15120706 | 222 | 1.95E-05 | CHG |
| | 5 | 15420814 | 86 | 4.47E-05 | CHG |

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|---|---|---|---|---|---|
| | 5 | 16811091 | 52 | 0.000142984 | CG |
| | 5 | 16929714 | 71 | 0.000303737 | CHG,CHH |
| | 5 | 17267296 | 343 | 2.34E-06 | CHG |
| | 5 | 17276625 | 306 | 0.000152296 | CHG |
| | 5 | 17577544 | 280 | 0.000374059 | CG |
| | 5 | 19358296 | 120 | 4.18E-07 | CG,CHG |
| | 5 | 19555694 | 155 | 2.44E-09 | CG |
| | 5 | 19648811 | 101 | 0.001037826 | CHG |
| | 5 | 20473165 | 238 | 2.08E-05 | CG |
| | 5 | 20839706 | 71 | 0.000170282 | CHG |
| | 5 | 21145373 | 289 | 9.30E-16 | CG |
| | 5 | 22186866 | 104 | 3.84E-06 | CHG |
| | 5 | 22703532 | 356 | 1.43E-07 | CHG |
| **Sperm Cell DMR** | 1 | 19668858 | 96 | 0.000373927 | CHH |
| | 1 | 24584011 | 195 | 8.24E-05 | CHG |
| | 2 | 726698 | 1186 | 7.71E-05 | CG |
| | 2 | 6164159 | 99 | 7.71E-05 | CHH |

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|---|---|---|---|---|---|
| | 2 | 10010408 | 298 | 8.24E-05 | CHH |
| | 2 | 12328902 | 203 | 8.24E-05 | CHG,CHH |
| | 3 | 16146242 | 160 | 8.24E-05 | CG,CHG,CHH |
| | 3 | 16281529 | 88 | 8.24E-05 | CHH |
| | 3 | 17358123 | 164 | 7.71E-05 | CHG,CHH |
| | 5 | 9175604 | 1027 | 3.23E-06 | CG |
| | 5 | 15006564 | 115 | 8.24E-05 | CG |
| **Vegetative Cell DMR** | 2 | 1089782 | 55 | 0.000197732 | CHH |
| | 2 | 5291300 | 88 | 0.000255115 | CHH |
| | 5 | 15561485 | 109 | 2.17E-07 | CG |

## 7.4 Appendix for Chapter 5

**Table 7.** DMR-flanking genes that differentially expressed in response to salt treatment

| Genes | lncRNA Present | Fold changes after salt treatment (150mM NaCl) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | shoot_ 0.5 hr | shoot_ 1 hr | shoot_ 3 hr | Shoot _6 hr | shoot_ 12 hr | shoot_ 24 hr | root_ 0.5 hr | root_ 1 hr | root_ 3 hr | root_ 6 hr | root_ 12 hr | Shoot _0.5 hr |
| AT1G03210 | No | -1.2 | -1.08 | -1.19 | -1.05 | -1.01 | -1.46 | -1.0 | -1.07 | -1.76 | -2.66 | -2.06 | -1.67 |
| AT1G07570 | Yes | -1.0 | -1.2 | -1.97 | -2.74 | -2.48 | -1.97 | 1.43 | 1.11 | 1.33 | 1.23 | 1.03 | 1.11 |
| AT1G07590 | Yes | 1.09 | 1.12 | 1.09 | 1.16 | 1.5 | 1.47 | 6.88 | 2.59 | 3.64 | 3.67 | 3.71 | 7.91 |
| AT1G10780 | No | 1.14 | -1.0 | 1.11 | 1.0 | 1.02 | -1.19 | -1.12 | -1.37 | -2.21 | -2.35 | -2.52 | -2.61 |
| AT1G11280 | Yes | 1.1 | 1.13 | -1.24 | -1.41 | -1.6 | -1.48 | -1.1 | -1.35 | -1.99 | -1.95 | -2.18 | -2.72 |
| AT1G26550 | No | -1.07 | -1.11 | 1.19 | 1.17 | 1.11 | 1.09 | -1.46 | -1.47 | -1.89 | -2.51 | -1.56 | -1.62 |
| AT1G27210 | Yes | 2.05 | -1.04 | 1.1 | -1.17 | -1.48 | -2.1 | 1.09 | 1.18 | -1.15 | -1.77 | -2.18 | -1.63 |
| AT1G31830 | Yes | -1.02 | 1.15 | 2.49 | 2.69 | 2.97 | 2.33 | -1.21 | -1.31 | -1.79 | -2.38 | -1.53 | -1.53 |
| AT1G33240 | Yes | 1.08 | -1.37 | -1.88 | -2.16 | -3.45 | -2.08 | 1.04 | -1.04 | -1.11 | 1.02 | -1.34 | -1.27 |
| AT1G42960 | No | 1.05 | 1.09 | 1.25 | 1.05 | 1.18 | 1.11 | -1.1 | -1.32 | -2.22 | -3.33 | -1.88 | -1.86 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AT1G47400 | Yes | -4.28 | -2.29 | 1.52 | 1.43 | 2.21 | 2.72 | -1.03 | 1.78 | 2.88 | 1.12 | 3.03 | 1.32 |
| AT1G51805 | Yes | -1.1 | 1.04 | -1.5 | -1.37 | -2.53 | -2.15 | -1.0 | 1.0 | 1.05 | -1.05 | 1.01 | 1.03 |
| AT1G52040 | No | 2.3 | 1.86 | 4.38 | 6.1 | 7.2 | 3.76 | -1.16 | -1.09 | -1.26 | -1.2 | -1.03 | -1.2 |
| AT1G52070 | Yes | -1.57 | -1.33 | -1.29 | -1.47 | -1.48 | -1.53 | -1.13 | -1.23 | -4.91 | -3.9 | -6.25 | -1.94 |
| AT1G54870 | Yes | 1.1 | 1.08 | 1.19 | 1.15 | 1.02 | 1.38 | 1.14 | 1.05 | 1.25 | 1.96 | 2.9 | 1.3 |
| AT1G54890 | No | 1.05 | 1.2 | -1.06 | 1.17 | 1.02 | -1.04 | -1.44 | -2.6 | -1.85 | 2.73 | 1.24 | 2.28 |
| AT1G59960 | No | -1.7 | -1.91 | -1.84 | -1.63 | -1.06 | -1.32 | -1.03 | -1.08 | -1.01 | -1.1 | -1.46 | -1.45 |
| AT1G60730 | Yes | -1.45 | -1.42 | -1.27 | -1.24 | -1.54 | -1.45 | 1.02 | 2.01 | 6.05 | 3.97 | 2.91 | 3.56 |
| AT1G61260 | Yes | 1.07 | -1.14 | -1.05 | -1.06 | 1.03 | -1.07 | 1.2 | 1.48 | 2.67 | 3.55 | 3.26 | 4.06 |
| AT1G63420 | No | 1.09 | 1.27 | 2.57 | 2.4 | 2.31 | 1.95 | -1.41 | -1.66 | -1.92 | -2.16 | -2.01 | -1.61 |
| AT1G64470 | Yes | 1.29 | 1.28 | 1.38 | 1.43 | 1.14 | 1.1 | 3.23 | 2.35 | 2.59 | 3.6 | 2.59 | 2.8 |
| AT1G66230 | Yes | -1.35 | -3.64 | -3.85 | -5.02 | -4.93 | -5.05 | 1.25 | 1.47 | -1.04 | 1.05 | 1.22 | 1.23 |
| AT1G68840 | Yes | -1.44 | -9.72 | -9.68 | -15.8 | -30.61 | -19.85 | 1.15 | 1.49 | 5.81 | 7.49 | 2.99 | 3.27 |
| AT1G71000 | Yes | 1.15 | 1.08 | 1.13 | 1.36 | 1.07 | -1.0 | -1.09 | 1.0 | 2.55 | 3.12 | 7.93 | 15.36 |
| AT1G71890 | No | -1.84 | -1.84 | -1.73 | -2.1 | -2.27 | -2.42 | -1.09 | -1.06 | -1.17 | 1.22 | 1.14 | 1.27 |
| AT1G72070 | Yes | -1.45 | -1.48 | -1.55 | -1.69 | -2.08 | -1.83 | 1.07 | 1.25 | 2.12 | 1.81 | 2.18 | 1.92 |
| AT1G73280 | No | 1.1 | 1.19 | 1.03 | 1.05 | 1.04 | 1.05 | -1.26 | -1.08 | -1.46 | -1.89 | -1.57 | -1.67 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AT2G02000 | No | -1.55 | -1.58 | -1.41 | -1.23 | 1.53 | 8.78 | 1.03 | 2.97 | 26.91 | 53.6 | 12.07 | 26.49 |
| AT2G02010 | Yes | -1.55 | -1.58 | -1.41 | -1.23 | 1.53 | 8.78 | 1.03 | 2.97 | 26.91 | 53.6 | 12.07 | 26.49 |
| AT2G02230 | Yes | 1.32 | 1.07 | 1.44 | 1.18 | 1.07 | 1.44 | 1.94 | 2.22 | 5.42 | 4.46 | 2.9 | 2.65 |
| AT2G04080 | No | 1.08 | 1.21 | 2.06 | 3.24 | 2.7 | 1.66 | 1.17 | -1 | 1.83 | 4.02 | 2.78 | 3.53 |
| AT2G07675 | No | 1.18 | -1.34 | -1.55 | -1.44 | -1.32 | 1.0 | 1.24 | 1.13 | -1.21 | -1.22 | 1.5 | 2.06 |
| AT2G07676 | No | -1.3 | -1.41 | -1.54 | -1.61 | -1.29 | -1.51 | -1.81 | -2.75 | -2.48 | -1.97 | -2.01 | -1.64 |
| AT2G14890 | Yes | 1.28 | 1.05 | 1.27 | 1.61 | 1.49 | 1.31 | -1.16 | -1.29 | -1.88 | -1.79 | -1.94 | -1.83 |
| AT2G15390 | No | -1.29 | -1.43 | -1.5 | -1.39 | -1.46 | -1.73 | -1.95 | -1.38 | 1.22 | 1.06 | -1.13 | -1.15 |
| AT2G18110 | Ye | -1.03 | -1.04 | -1 | -1.03 | 1.05 | 1.04 | 1.14 | 1.05 | -1.4 | -1.96 | -1.48 | -1.52 |
| AT2G18330 | Yes | -1.09 | -1.16 | -1.78 | -1.5 | -1.14 | -1.24 | -1.35 | -1.55 | -1.83 | -1.5 | -2.1 | -2.06 |
| AT2G21650 | No | 1.04 | 1.13 | -2.28 | -14.82 | -18.15 | -16.76 | -1.25 | -1.24 | -1.81 | -1.67 | -1.49 | 1.28 |
| AT2G21900 | No | 1.1 | 1.31 | 1.29 | 1.15 | 1.01 | 1.24 | 1.27 | 1.8 | 7.21 | 14.04 | 2.63 | 4.95 |
| AT2G24762 | Yes | 1.72 | -1.84 | -1.73 | 1.43 | 1.42 | 1.25 | -1.68 | -2.02 | 1.16 | 1.16 | -1.05 | -1.15 |
| AT2G25680 | Yes | -1.54 | -1.54 | -1.69 | -2.02 | -2.09 | -1.42 | -1.0 | -1.06 | -1.27 | -1.04 | -1.13 | 1.39 |
| AT2G26440 | Yes | -1.84 | -1.43 | -3.78 | -1.22 | -2.52 | -3.46 | -1.02 | -1.36 | -1.72 | -2.41 | -3.04 | -2.82 |
| AT2G27530 | Yes | -1.09 | -1.09 | -2 | -1.92 | -1.54 | -1.49 | -1.15 | -1.15 | -1.68 | -2.28 | -2.03 | -1.63 |
| AT2G27730 | No | -1.05 | -1.02 | -1.02 | 1.07 | 1.22 | 1.05 | -1.14 | -1.15 | -1.69 | -2.14 | -1.6 | -1.56 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AT2G27740 | No | 1.03 | 1.13 | 1 | 1.02 | 1.21 | 1.46 | -1.14 | -1.03 | -1.7 | -1.96 | -1.54 | -1.5 |
| AT2G30070 | Yes | -1.1 | 1.12 | -1.24 | -1.28 | 1.28 | -1.16 | 1.82 | 2.0 | 3.19 | 4.21 | 3.25 | 2.17 |
| AT2G32190 | Yes | -1.65 | -1.93 | -2.05 | -2.11 | -1.96 | 3.11 | 1.5 | 3.23 | 10.11 | 17.05 | 5.83 | 7.36 |
| AT2G33370 | Yes | -1.05 | -1.13 | -1.42 | -2.07 | -1.78 | -2.96 | -1.13 | -1.22 | -1.71 | -2.46 | -2.21 | -2.2 |
| AT2G33380 | Yes | 1.22 | 2.05 | 27.11 | 18.48 | 19.38 | 27.57 | 1.5 | -1.09 | 1.66 | 2.39 | 8.22 | 9.07 |
| AT2G34060 | Yes | -1.01 | -1.14 | -1.11 | -1.9 | -2.31 | -2.16 | 1.31 | -1.05 | -1.16 | -1.08 | -1.05 | -1.39 |
| AT2G34070 | Yes | -2.26 | -3.12 | -3.03 | -2.6 | -1.61 | -1.4 | -1.03 | 1.29 | 2.04 | 2.67 | 1.24 | 1.18 |
| AT2G35940 | Yes | -1.06 | 1.37 | 1.96 | 1.45 | 1.9 | 2.17 | 1.46 | 1.88 | 1.69 | 1.69 | 2.96 | 3.05 |
| AT2G38390 | No | -2.06 | -2.06 | -1.03 | -1.78 | -1.42 | -2.26 | -1.06 | -1.11 | -1.26 | -1.74 | -1.05 | -1.05 |
| AT2G34060 | Yes | 1.1 | 1.06 | -1.0 | 1.03 | 1.45 | 1.07 | 1.39 | 1.13 | 1.8 | 2.01 | 1.62 | 2.11 |
| AT2G41990 | Yes | 1.15 | 1.4 | -1.24 | -1.31 | -1.94 | -2.44 | -1.01 | -1.2 | -1.22 | 1.05 | 1.06 | -1.18 |
| AT3G03420 | Yes | -1.22 | -1.34 | -1.6 | -1.59 | -1.2 | -1.19 | -1.33 | -1.33 | -1.29 | -1.42 | -1.96 | -1.99 |
| AT3G04240 | Yes | -1.17 | -1.02 | 1.55 | 1.4 | 1.64 | 1.84 | 1.13 | 1.41 | 2.54 | 3.78 | 2.52 | 3.09 |
| AT3G07000 | No | -1.1 | -1.06 | -1.2 | -1.27 | -1.21 | -1.24 | -1.52 | -1.81 | -1.6 | 1.3 | -1.43 | -1.86 |
| AT3G07010 | Yes | 1.98 | 2.26 | -1.35 | -1.16 | -1.14 | -2.83 | 1.04 | -1.28 | -1.2 | 1.01 | -1.67 | 1.16 |
| AT3G07130 | Yes | -1.15 | 1.1 | 1.06 | -1.02 | -1.13 | 1.03 | -1.49 | -1.42 | -3.08 | -4.23 | -2.1 | -2.1 |
| AT3G18040 | No | 1.11 | 1.06 | 1.01 | 1.18 | 1.27 | 1.31 | 1.1 | 1.17 | 1.07 | 1.93 | 1.99 | 1.88 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AT3G19480 | Yes | -1.07 | -0.14 | -1.76 | -2.23 | -2.02 | -2.3 | -1.14 | -1.21 | -1.44 | -1.4 | 1.07 | -1.11 |
| AT3G21870 | Yes | -1.07 | -1.51 | -1.92 | -5.53 | -5.27 | -4.34 | -1.02 | -1.07 | -1.3 | -1.22 | -1.11 | -1.07 |
| AT3G22160 | Yes | -1.39 | -1.37 | -1.38 | -1.61 | -1.63 | -1.69 | 1.07 | 1.8 | 5.17 | 6.18 | 2.46 | 3.24 |
| AT3G25070 | Yes | -1.04 | -1.09 | -1.86 | -2.06 | -2.03 | -2.4 | -1.06 | -1.15 | 1.51 | 1.63 | 1.09 | 1.02 |
| AT3G25110 | Yes | -1.01 | 1.18 | 1.55 | 2.18 | 2.18 | 2.71 | -1.14 | -1.09 | -1.59 | -1.92 | -1.12 | -1.12 |
| AT3G43250 | Yes | 1.04 | -1.01 | -1.1 | -1.1 | -1.04 | -1.0 | 1.08 | 1.27 | 2.6 | 5.81 | 1.43 | 1.13 |
| AT3G45140 | Yes | 1.07 | 1.11 | 2.23 | 5.67 | 8.4 | 3.62 | 1.04 | 1.1 | -1.07 | 1.02 | 1.03 | 1.12 |
| AT3G46010 | Yes | -1.06 | 1.09 | 1.07 | 1.23 | 1.07 | -1.11 | -1.25 | -1.44 | -2.02 | -2.49 | -1.77 | -1.73 |
| AT3G49380 | No | 1.03 | -1 | 1.07 | 1.18 | -1.01 | -1.05 | 1.29 | 1.14 | 1.31 | 2.42 | 1.76 | 1.31 |
| AT3G56200 | Yes | 1.14 | -1.69 | -1.18 | 1.38 | 1.46 | 2.77 | -1.08 | 1.61 | 2.82 | 4.81 | 2.29 | 1.87 |
| AT4G00955 | No | -1.66 | -1.46 | -2.13 | -1.89 | -2.46 | -1.82 | -1.28 | -1.26 | -1.24 | -1.23 | -1.08 | 1.02 |
| AT4G01660 | No | -1.31 | -1.59 | -1.79 | -1.18 | 1.07 | 1.64 | -1.42 | -0.139 | -1.46 | -1.45 | -1.19 | -1.15 |
| AT4G03030 | Yes | 1.14 | -1.03 | 1.76 | 1.86 | 1.9 | 2.09 | 1.08 | 1.24 | 1.23 | -1.02 | 1.3 | 1.28 |
| AT4G04220 | Yes | -1.89 | -1.47 | -1.76 | -1.28 | -1.65 | -1.6 | 1.02 | 1.17 | 1.49 | 2.33 | 1.66 | 1.6 |
| AT4G04750 | No | 1.15 | 1.15 | 1.81 | 1.62 | 2.51 | 2.25 | -1.08 | -1.25 | -1.44 | -1.2 | 1.39 | -1.2 |
| AT4G08850 | Yes | -1.85 | -1.64 | -4.45 | -2.37 | -2.68 | -2.44 | 1.01 | 1.47 | 3.25 | 4.45 | 2.42 | 5.66 |
| AT4G09030 | Yes | -2.28 | -3.01 | -1.62 | -1.09 | 1.32 | 1.92 | 1.51 | 2.2 | 3.44 | 4.5 | 3.38 | 2.7 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AT4G10120 | Yes | -1.21 | -1.5 | -2.65 | -5.64 | -3.04 | -2.44 | 1.04 | -1 | -1.05 | -1.15 | 1.1 | 1.13 |
| AT4G14240 | Yes | -1.37 | -1.16 | -1.38 | -1.24 | -1.24 | 1.13 | -1.13 | -1.28 | -1.91 | -1.68 | -1.39 | -1.03 |
| AT4G14365 | Yes | -1.94 | -2.35 | -2.91 | -2.33 | -3.3 | -2.41 | 2.35 | 2.9 | 13.02 | 18 | 4.76 | 8.1 |
| AT4G15440 | Yes | 1.26 | 1.86 | 1.56 | 2.16 | 13.14 | 4.44 | 1.18 | 1.29 | 1.82 | 2.04 | 1.32 | 1.15 |
| AT4G19680 | No | -1.01 | -1.04 | -1.18 | -1.14 | -1.05 | 1.02 | -1.65 | -1.53 | -1.84 | -2.0 | -1.89 | -1.42 |
| AT4G19690 | Yes | -1.12 | 1.02 | -1.15 | -1.04 | -1.03 | 1.0 | -1.91 | -1.54 | -15.05 | -54.56 | -90.01 | -19.9 |
| AT4G20000 | Yes | -2.16 | -2.5 | -2.12 | -2.55 | -2.59 | -2.19 | 1.7 | 3.6 | 10.66 | 10.04 | 2.64 | 2.17 |
| AT4G20010 | No | -1.2 | -1.27 | -1.54 | -1.72 | -1.29 | -1.41 | -1.06 | 1.13 | 2.87 | 2.76 | 1.26 | 1.22 |
| AT4G21090 | No | -1.25 | -1.29 | -2.05 | -1.66 | -1.35 | -1.2 | -1.4 | -1.29 | -1.16 | -1.22 | -1.38 | -1.49 |
| AT4G21903 | No | -1.09 | -1.11 | 2.07 | 2.09 | 4.22 | 2.67 | 1.35 | 2.19 | 1.86 | 1.33 | 1.21 | 1.46 |
| AT4G21910 | No | -1.09 | -1.11 | 2.07 | 2.09 | 4.22 | 2.67 | 1.35 | 2.19 | 1.86 | 1.33 | 1.21 | 1.46 |
| AT4G23570 | No | -1.56 | -1.72 | -2.06 | -2.13 | -1.91 | -1.52 | 1.06 | -1.03 | 1.25 | 2.38 | 1.73 | 2.34 |
| AT4G29285 | No | -1.04 | 1.02 | -1.05 | 1.02 | -1.03 | -1 | -1.11 | -1.18 | 2.2 | 3.34 | 1.02 | -1.08 |
| AT4G33280 | No | 1.04 | 1.11 | -1.04 | -1.05 | -1.05 | -1.15 | 1.19 | 1.03 | -1.05 | 2.65 | 1.84 | 2.67 |
| AT4G33300 | Yes | 1.21 | -1.08 | -1.16 | 1.15 | -1.2 | -1.92 | 1.42 | 1.46 | 1.5 | 1.44 | 1 | -1.05 |
| AT5G03670 | Yes | -1.05 | -1.19 | 1 | 1.01 | -1.19 | -1.04 | 1.47 | 1.76 | 2.04 | -1.13 | 1.17 | 1.33 |
| AT5G10190 | Yes | 1.0 | 1.09 | -1.04 | 1.11 | 1.13 | 1.08 | 1.52 | 1.66 | 2.22 | 2.46 | 1.72 | 1.57 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AT5G18890 | No | 1.59 | 1.69 | 2.18 | 1.61 | 1.15 | 2.9 | -1.05 | 1.63 | 1.89 | 1.53 | 1.03 | 1.03 |
| AT5G22520 | Yes | 1.11 | 1.09 | 1.05 | 1.04 | 1.06 | 1.05 | 2.32 | 8.42 | 18.35 | 18.11 | 3.2 | 2.87 |
| AT5G22530 | Yes | 1.11 | 1.09 | 1.05 | 1.04 | 1.06 | 1.05 | 2.32 | 8.42 | 18.35 | 18.11 | 3.2 | 2.87 |
| AT5G23190 | Yes | 1.04 | -1 | -1.05 | 1.15 | 1.04 | -1.07 | -1.14 | -1.16 | -1.27 | -2.48 | 1.37 | 1.4 |
| AT5G24655 | No | -1.08 | -2.51 | -1.07 | 1.38 | 1.86 | 2.92 | 1.12 | 1.31 | 1.84 | 12.38 | 3.89 | 2.48 |
| AT5G25910 | Yes | -1.02 | 1.03 | 1.02 | 1.01 | -1.13 | 1.01 | 1.13 | 1.03 | 1.43 | 2.01 | 1.37 | 1.38 |
| AT5G25930 | Yes | -1.1 | -1.7 | -1.73 | -1.5 | -1.99 | -1.9 | 1.52 | 3.77 | 9.7 | 11.11 | 4.54 | 5.12 |
| AT5G27140 | Yes | 1.08 | -1.04 | -2 | -1.7 | -1.4 | -1.18 | 1.16 | -1.29 | -2.09 | -2.08 | -1.5 | -1.18 |
| AT5G27420 | Yes | -1.16 | -1.98 | -1.61 | -1.96 | -2.36 | -2.09 | 2.28 | 6.75 | 23.29 | 7.11 | 9.57 | -1.49 |
| AT5G27430 | Yes | -1.07 | -1.17 | -1.25 | -1.29 | 1.03 | -1.13 | -1.5 | -1.53 | -1.66 | -2.44 | -2.08 | -1.91 |
| AT5G28610 | No | -1.11 | 1.16 | 1 | 1.09 | 1.01 | -1.09 | 2.6 | 5.13 | 16.72 | 16.92 | 3.97 | 2.86 |
| AT5G42020 | Yes | -1.59 | -1.54 | -2.54 | -1.22 | 1.16 | 1.05 | -1.25 | -1.34 | -2.8 | -3.33 | -2.0 | -1.41 |
| AT5G42030 | Yes | 1.04 | 1.08 | 1.87 | 1.47 | 1.38 | 1.25 | -1.1 | -1.18 | -1.46 | -3.13 | -1.73 | -1.94 |
| AT5G43060 | Yes | -1.03 | -1.01 | -1.12 | 1.16 | 1.28 | -1.99 | 1.05 | -1.04 | -1.62 | -1.65 | 1.18 | 1.08 |

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